



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁴ : C12N 9/16, C07K 7/08 A61K 37/02, 39/392, C12N 15/00		A1	(11) International Publication Number: WO 89/09818 (43) International Publication Date: 19 October 1989 (19.10.89)
<p>(21) International Application Number: PCT/US89/01418</p> <p>(22) International Filing Date: 11 April 1989 (11.04.89)</p> <p>(30) Priority data: 181,893 15 April 1988 (15.04.88) US 219,491 12 July 1988 (12.07.88) US</p> <p>(71) Applicant: BIOGEN, INC. [US/US]; 14 Cambridge Center, Cambridge, MA 02142 (US).</p> <p>(72) Inventors: KRAMER, Ruth, M. ; 3740 Governor's Road, Golden Hill, IN 46208 (US). PEPINSKY, R., Blake ; 69 Parker Street, Watertown, MA 02172 (US). HESSION, Catherine ; 96 Fountain Lane, South Weymouth, MA 02190 (US).</p> <p>(74) Agents: HALEY, James, F., Jr. et al.; Fish & Neave, 875 Third Avenue, New York, NY 10022-6250 (US).</p>		<p>(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent).</p> <p>Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	

(54) Title: PROCESSES FOR PURIFYING PHOSPHOLIPASE A₂ AND PRODUCING PHOSPHOLIPASE A₂-LIKE POLYPEPTIDES

(57) Abstract

This invention relates to processes for purifying acid stable phospholipase A₂. Specifically, the invention relates to processes for the purification of phospholipase A₂ from biological sources, such as non-pancreatic human sources which contain very small amounts of that enzyme. More specifically, this invention relates to the purification and characterization of phospholipase A₂ from human platelets and from human rheumatoid synovial fluid. This invention also relates to polypeptides corresponding to at least a portion of the amino terminal amino acid sequence of human platelet and rheumatoid synovial fluid phospholipase A₂ and antibodies thereto, as well as antibodies to purified, intact acid-stable phospholipase A₂ for use in the treatment or diagnosis of inflammation and tissue injury associated with various diseases. And this invention relates to DNA sequences which encode these polypeptides. This invention further relates to methods for producing phospholipase A₂ in hosts transformed with recombinant DNA molecules comprising those DNA sequences.

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-1-

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PROCESSES FOR PURIFYING
PHOSPHOLIPASE A₂ AND PRODUCING
PHOSPHOLIPASE A₂-LIKE POLYPEPTIDES

TECHNICAL FIELD OF INVENTION

10 This invention relates to processes for purifying acid stable phospholipase A₂. Specifically, the invention relates to processes for the purification of phospholipase A₂ from biological sources, such as non-pancreatic human sources which

15 contain very small amounts of that enzyme. More specifically, this invention relates to the purification and characterization of phospholipase A₂ from human platelets and from human rheumatoid synovial fluid. This invention also relates to polypeptides

20 corresponding to at least a portion of the amino terminal amino acid sequence of human platelet and rheumatoid synovial fluid phospholipase A₂, and antibodies thereto, as well as antibodies to purified, intact, acid-stable phospholipase A₂ for use in the

25 treatment or diagnosis of inflammation and tissue injury associated with various diseases. This invention further relates to DNA sequences which encode human inflammatory phospholipase A₂ and methods for producing phospholipase A₂ in hosts transformed with

-2-

recombinant DNA molecules comprising those DNA sequences.

BACKGROUND ART

5 Phospholipases A_2 (phosphatide 2-acylhydrolyase, EC 3.1.1.4, PLA₂) are a family of lipolytic proteins that specifically cleave the acyl ester linkage at the sn-2 position of glycerophospholipids. These enzymes are ubiquitous and are present in virtually every cell type from bacteria to man.

10 Nearly all of the phospholipases A_2 studied to date have a molecular weight of between 10 and 15 kilodaltons, but they differ substantially in amino acid sequence. Secretory phospholipases A_2 may be divided into two categories: digestive (produced and secreted by digestive organs, such as the pancreas), and inflammatory (produced and secreted by inflammatory cells, such as platelets or neutrophils, or found in inflammatory fluids, such as rheumatoid synovial fluid).

15 In mammals, phospholipase A_2 is found in abundant quantities in the pancreas. Other cellular and extracellular mammalian phospholipases A_2 are found in much smaller amounts. In humans, non-pancreatic phospholipases A_2 have been found in seminal plasma, synovial fluid, septic shock serum, and bronchoalveolar lavage fluid of alveolar proteinosis (P. Vadas and W. Pruzanski, "Biology of Disease. Role of Secretory Phospholipases A_2 in the Pathobiology of Disease", Lab. Invest., 55, pp. 391-404 (1986)). Most of the mammalian phospholipases A_2 are acid-stable and all are calcium-dependent to varying degrees. To date, with one exception, non-pancreatic phospholipases A_2 demonstrate no immunological cross-reactivity with pancreatic phospholipase A_2 (J. G. N. DeJung et al. "Monoclonal Antibodies Against an Intracellular Phospholipase

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A_2 from Rat Liver and their Cross-Reactivity with Other Phospholipases A_2 ", Eur. J. Biochem., 164, pp. 129-35 (1987)).

Intracellular phospholipases A_2 are involved in various physiological functions, including membrane phospholipid turnover, repair of membrane peroxidation damage, transmembrane signaling, cell membrane dynamics and generation of lipid mediators. The control and regulation of phospholipases A_2 is complex and involves many factors, including free calcium concentration, molecular entities involved in transmembrane signaling, and the physicochemical state of the phospholipid substrate (H. van den Bosch in Comprehensive Biochemistry, vol. 4, pp. 313-57, J. N. Hawthorne and G. B. Ansell, eds., Elsevier Amsterdam (1982)).

Phospholipases A_2 are also known to mediate a variety of pathophysiological conditions through the products of protein catalysis -- lysophospholipids and arachidonic acid. (J. Chang et al., "Phospholipase A_2 : Function and Pharmacological Regulation", Biochem. Pharmacol., 36, pp. 2429-36 (1987); P. Vadas and W. Pruzanski, Lab. Invest., 55, pp. 391-404 (1986); A. A. Farooqui et al., "Phospholipases, Lysophospholipases, and Lipases and Their Involvement in Various Diseases", Neurochem. Path., 7, pp. 99-128 (1987)). Lysophospholipids are fusogenic and cytotoxic. Subsequent metabolism of phospholipase A_2 catalytic products by certain protein cascades leads to several potent, biologically active substances. These include prostaglandins, hydroxylated fatty acids, leukotrienes and platelet activating factor, all of which have been implicated in inflammation or hypersensitivity, or both. Many studies have indicated that phospholipases A_2 play important roles in inflammation and tissue injury associated with various diseases, such as viral and bacterial infections,

skin and connective tissue diseases, such as psoriasis, 5 gastrointestinal disorders, such as pancreatitis and ulcers, ischemias, myocardial infarction, atherosclerosis, pulmonary dysfunctions, such as asthma,

acute respiratory distress syndrome and alveolar proteinosis, septic shock, thrombosis, multiple sclerosis, demyelinating diseases and rheumatoid arthritis. Thus, inhibition or inactivation of pathogenic phospholipase A_2 activity is of clinical 10 importance.

Pancreas-derived phospholipase A_2 has been purified, sequenced and structurally defined (H. M. Verheij et al., "Structure and Function of Phospholipase A_2 ", Rev. Physiol. Biochem. Pharmacol., 91, 15 pp. 91-203 (1981)). The protein is produced in the form of an inactive precursor which is stored in secretory granules. Once secreted in the intestine the precursor is activated by limited tryptic proteolysis, leading to the formation of the active phospholipase and a small polypeptide. No evidence 20 for such a precursor has been obtained with respect to inflammatory phospholipases A_2 .

To date, only three mammalian non-pancreatic phospholipases A_2 have been purified to homogeneity 25 as evidenced by internal sequence analysis. These include phospholipase A_2 from rabbit inflammatory peritoneal exudate (S. Forst et al., "Structural and Functional Properties of a Phospholipase A_2 Purified from an Inflammatory Exudate", Biochemistry, 25, pp. 30 8381-85 (1986)), phospholipase A_2 from secreted rat platelets (M. Hayakawa et al., "Amino Acid Composition and NH_2 -Terminal Amino Acid Sequence of Rat Platelet Secretory Phospholipase A_2 ", J. Biochem., 101, pp. 1311-14 (1987)), and phospholipase A_2 from rat 35 inflammatory peritoneal exudate (H. W. Chang et al., "Purification and Characterization of Extracellular Phospholipase A_2 from Peritoneal Cavity of Caseinate-

-5-

Treated Rat", J. Biochem., 102, pp. 147-54 (1987)).

Many attempts have been made to isolate phospholipases A₂ from human non-pancreatic sources, but none has succeeded in purifying these proteins to homogeneity.

5 Thus, to date, the primary structure of these phospholipases A₂ has not been identified.

The need exists for a process for purifying acid stable phospholipases A₂ to homogeneity which, advantageously, also permits purification of

10 the protein from human non-pancreatic biological sources.

DISCLOSURE OF THE INVENTION

The present invention solves the problems referred to above by providing processes for purifying acid stable phospholipases A₂ from biological sources, such as mammalian cells and extracellular fluid, plant cells, insect cells, yeast and other fungi, and bacteria. Specifically, these processes permit the purification of inflammatory phospholipases A₂ to homogeneity from human, non-pancreatic sources such as platelets and rheumatoid synovial fluid. The phospholipases A₂ purified from human platelets and rheumatoid synovial fluid by the processes of this invention are characterized by a common amino-terminal amino acid structure, which differs significantly from that of pancreatic-derived phospholipase A₂. According to one embodiment of this invention, phospholipase A₂ purified from rheumatoid synovial fluid exhibits an inflammatory activity that is at least 100-fold greater than that of the pancreatic-derived protein in an assay of inflammatory action that measures formation of paw edema in the rat after subplant or injection of purified phospholipase A₂ (S. Brain et al, "Action 30 of Phospholipase A on Mast Cell Histamine Release

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-6-

and Paw Edema in the Rat", Brit. J. Pharmacol., 59, pp. 440-41 (1977)).

This invention also relates to the production of polypeptides corresponding to at least a portion of the amino acid sequence of these inflammatory phospholipases A₂. Such polypeptides, as well as antibodies to these polypeptides and antibodies to intact, purified phospholipases A₂, are useful for pharmacological, therapeutic and diagnostic purposes. In addition, DNA sequences encoding all or part of the deduced amino acid sequence of these phospholipases A₂ are useful as diagnostics for the evaluation and monitoring of diseases, or as probes for the isolation of cDNA or genomic clones coding for human inflammatory phospholipases A₂.

This invention also relates to the production of phospholipase A₂ using recombinant techniques. In such a process, DNA sequences coding for the phospholipases A₂ of this invention, recombinant DNA molecules characterized by those sequences and unicellular hosts transformed with those molecules are employed to produce phospholipases A₂ by culture of those transformed hosts.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the elution profile of a partially purified preparation of human platelet phospholipase A₂ off of a cation exchange chromatography column.

Figure 2 depicts the elution profile of a partially purified preparation of human platelet phospholipase A₂ off of a gel filtration column.

Figure 3, panel A, depicts the elution profile of a partially purified preparation of human platelet phospholipase A₂ off of a reverse phase HPLC column. Figure 3, panel B, depicts the corres-

ponding phospholipase A₂ activity of the eluted fractions.

5 Figure 4 depicts in tabular form the purification of phospholipase A₂ from isolated human platelets.

Figure 5 depicts an Immobilon blot of the human platelet phospholipase A₂-containing fractions collected following HPLC.

10 Figure 6 depicts the elution profile of a partially purified preparation of human rheumatoid synovial fluid phospholipase A₂ off of a cation exchange chromatography column.

15 Figure 7 depicts the elution profile of a partially purified preparation of human rheumatoid synovial fluid phospholipase A₂ off of a gel filtration column.

20 Figure 8, panel A, depicts the elution profile of a partially purified preparation of human rheumatoid synovial fluid phospholipase A₂ off of a reverse phase HPLC column. Figure 8, panel B, depicts the corresponding protein activity of the eluted fractions.

25 Figure 9 depicts in tabular form the purification of phospholipase A₂ from human rheumatoid synovial fluid

Figure 10 depicts an Immobilon blot of the human rheumatoid synovial fluid phospholipase A₂-containing fractions collected following HPLC.

30 Figure 11A depicts portions of sequencing plasmid pNN01. Figure 11B depicts the restriction map of PSQ 130.

35 Figure 12 depicts the DNA sequence of 6.2 kb HindIII fragment of PLA₂ 8.5 EMBL3 and the amino acid sequence of human inflammatory phospholipase A₂ derived therefrom.

Figure 13 depicts a comparison of the amino acid sequences of bovine pancreatic phospholipase

-8-

A_2 , phospholipase A_2 from C. atrox venom, and the derived amino acid sequence from the 6.2 kb HindIII fragment of PLA_2 8.5 EMBL3.

5 Figure 14A depicts schematically the synthesis of BG368 from BG312. Figure 14B depicts the restriction map of PLA_2 6.2 BG368 3(+). Figure 14C depicts the restriction map of PLA_2 3.8 BG341(+). Figure 14D depicts the restriction map of BG341.

10 Figure 15A depicts a restriction map of pJODS. Figure 15B depicts a restriction map of PLA_2 3.8 JODS.

DETAILED DESCRIPTION OF THE INVENTION

15 This invention relates to processes for isolating and purifying acid stable phospholipases A_2 from biological sources. According to this invention, phospholipases A_2 may be purified to homogeneity as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and amino-terminal amino acid sequence analysis. This invention also 20 relates to polypeptides which are characterized by at least a portion of the amino terminal 19 amino acids of human inflammatory phospholipase A_2 purified from platelets and rheumatoid synovial fluid and to the DNA sequences which encode them.

25 Generally, one embodiment of the process of this invention begins with the step of acid-extracting the protein from a biological source, such as sonicated platelets or rheumatoid synovial fluid. The acid in the extract is then exchanged 30 for a buffer suitable for cation exchange chromatography and the preparation contacted with a cation exchange resin. The phospholipase A_2 is then eluted from the resin, concentrated and further purified away from dissimilar molecular weight contaminants 35 by molecular sizing. Fractions containing phospholipase A_2 activity are purified to homogeneity by

reverse-phase HPLC. According to another embodiment, the process of this invention further comprises the step of electrophoresing said phospholipase A₂ and transferring said electrophoresed phospholipase A₂ 5 to a solid support.

According to an alternate embodiment of this invention, which permits the isolation of an intracellularly located phospholipase A₂, the process comprises the further initial step of extracting or 10 releasing the protein from the cell. This may be achieved by any of a number of well-known lysing techniques, such as sonication, homogenization, French press, chemical lysis or enzymatic lysis. Mechanical lysis techniques are preferable, because they do not 15 introduce any extraneous proteins or organic chemicals into the phospholipase A₂ preparation. The most preferred method of lysis is sonication.

Acid extraction of the protein according to this invention may be achieved with any acid of 20 any concentration having a pH below about 4.5. Preferably, the acid is a mineral acid, such as hydrochloric acid, phosphoric acid or sulfuric acid and has a pH of about 1.0. The most preferable acid is sulfuric acid at a concentration of about 0.18 N, 25 which gives a pH of about 1.

In order to perform cation exchange chromatography on the phospholipase A₂ preparation, the protein should be in a buffer that is compatible with the cation exchange resin. Numerous methods of 30 achieving buffer exchange are known in the art, including dialysis, ultrafiltration and desalting. Because the phospholipase A₂ is a small protein of about 13,000 daltons, dialysis or ultrafiltration must be carried out using a membrane of suitably 35 small pore size. The most preferred method of buffer exchange is dialysis using a membrane that has a molecular weight cutoff of about 3500 daltons.

Various cation exchange resins that are commercially available may be used in the processes of this invention. Examples of some of these resins are Fast S, Mono S, CM-Sepharose, SP-Sepharose and phosphate-cellulose. The preferred properties of the resin used in the process of this invention are high flow rate, the ability to bind phospholipase A_2 at the cation concentration of initial contact and the ability to release phospholipase A_2 under higher ionic conditions. Cation exchange may be performed batchwise, or preferably in a column. The most preferable conditions for cation exchange are initially binding the protein to a Fast S column in 200 mM NaCl, 50 mM acetate, pH 4.5 and eluting the protein with a linear gradient of 200 mM - 2 M NaCl. Using these conditions, the protein will elute at about 1 M NaCl.

Active fractions from cation exchange may be concentrated by any standard technique, preferably one which does not concurrently concentrate ions. The most preferable means of concentration is ultrafiltration using a membrane with a low molecular weight cutoff, such as a YM 5 membrane (Amicon).

Any one of a number of commercially available molecular sizing chromatography resins may be employed in the processes of this invention. Preferably, the resin will be such that the phospholipase A_2 will elute in the included volume. In this manner, the majority of higher molecular weight contaminants will be removed by elution in the void volume of the column. Among the preferred molecular sizing resins are Biogel P30, Biogel P60, Sephadex G-25, Sephadex G-50, Sephadex G-75 and Utragel AcA54. The most preferred resin is Sephadex G-50 superfine.

Active fractions are further purified by reverse phase HPLC. Any hydrophobic resin that is

compatible with HPLC may be used with the process of this invention. Examples of preferred resins are C18, C8, C4, C3, and phenyl. The most preferred resin is C4.

5 This invention also relates to phospholipases A₂ produced according to the above described processes. Phospholipase A₂ is most preferably characterized by enzymatic activity. For example, phospholipase A₂ cleaves and releases into the assay 10 supernatant [³H]-oleic acid from autoclaved, [³H]-oleic acid-labelled E.coli. Other phospholipase A₂ substrates include, but are not limited to, phosphatidylcholine and phosphatidylethanolamine.

15 In addition, various methods of substrate dispersion, such as sonication, solubilizing in organic solvents and mixing with detergents, as well as alternate assay conditions may be employed to characterize phospholipase A₂. All of these assay methods are well-known in the art (L. R. Ballou and 20 W. Y. Cheung, "Marked Increase of Human Platelet Phospholipase A₂ Activity In Vitro and Demonstration of an Endogenous Inhibitor", Proc. Natl. Acad. Sci. USA, 80, pp. 5203-07 (1983); R. M. Kramer et al., "Solubilization and Properties of Ca²⁺- Dependent 25 Human Platelet Phospholipase A₂", Biochim. Biophys. Acta, 878, pp. 394-403 (1986); M. A. Clark et al., "Leukotriene D₄ Treatment of Bovine Aortic Endothelial Cells and Murine Smooth Muscle Cells in Culture Results in an Increase in Phospholipase A₂ 30 Activity", J. Biol. Chem., 261, pp. 10713-18 (1986); L. A. Loeb and R. W. Gross, "Identification and Purification of Sheep Platelet Phospholipase A₂ Isoforms", J. Biol. Chem., 261, pp. 10467-70 (1986)).

35 Alternatively, phospholipase A₂ may be characterized by its reaction with a specific anti-

-12-

body in assays well-known in the art such as ELISA, Western Blots and immunoprecipitation.

Phospholipase A₂ purified according to this invention may be used to raise monoclonal or 5 polyclonal antibodies. It may also be cleaved with various endo- and exopeptidases to produce the polypeptides of this invention. As demonstrated in the following examples, such purified protein was used as a source of amino acid sequence data to permit 10 the synthesis of specific polypeptides which elicit site-specific anti-phospholipase A₂ antibodies. The amino acid sequence data was then employed to obtain nucleotide probes useful in isolating and selecting 15 a DNA sequence encoding phospholipase A₂ from a genomic or cDNA library.

The amino acid sequence of phospholipase A₂ purified by the processes described above may be obtained by directly sequencing the material recovered from reverse phase HPLC according to this invention. More preferably, and according to an alternate embodiment of the present invention, the phospholipase A₂ is first subjected to discontinuous 20 SDS-polyacrylamide gel electrophoresis (U. K. Laemmli, "Cleavage of Structural Proteins During the 25 Assembly of the Head of Bacteriophage T4, Nature, 227, pp. 680-85 (1970)). This allows the protein to be separated from other potential protein contaminants. Following electrophoresis, the separated protein(s) are transferred to a solid support. The 30 solid support should be compatible with a protein sequencer. Preferred solid supports are activated glass filters or, more preferably, polyvinylidene difluoride ("PVDF") membranes. The transfer of 35 electrophoresed phospholipase A₂ to the support may be achieved by capillary transfer, or more preferably, by electrophoretic transfer. Following transfer, proteins may be visualized with any of a

number of well-known protein stains, such as Coomassie Blue R-250. The major phospholipase A₂ band is excised from the membrane and subjected to protein sequencing.

5 Protein sequencing may be achieved by standard techniques, preferably using automated Edman degradation, such as with an Applied Biosystems 470A gas phase sequencer. The amino terminal 19 amino acids of both human rheumatoid

10 synovial fluid phospholipase A₂ and human platelet phospholipase A₂ purified according to this invention are both characterized by the amino acid sequence: H₂N-Asn-Leu-Val-Asn-Phe-His-Arg-Met-Ile-Lys-Leu-Thr-Thr-Gly-Lys-Glu-Ala-Ala-Leu. Such 100% homology

15 suggests that other human inflammatory phospholipases A₂ may also contain this sequence. This amino terminal sequence differs from that of any phospholipase A₂ that has been purified previously from either human or non-human sources.

20 Due to limitations in the number of amino acids that can be accurately sequenced by standard techniques, the entire amino acid sequence of phospholipase A₂ is most preferably derived from the nucleotide sequence of a full-length human inflammatory phospholipase A₂ DNA or cDNA clone. The amino acid sequence obtained from the purified natural product has been used to confirm the identification of phospholipase A₂ clones isolated according to the processes of the present invention.

25 This invention also relates to polypeptides which correspond in amino acid sequence to at least a portion of the amino terminal 19 amino acids of human inflammatory phospholipase A₂. These polypeptides may be used to immunize animals and raise

30 specific antibodies. Antibodies to small, weakly immunogenic polypeptides may be elicited by cross-linking the polypeptide to a carrier prior to injec-

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-14-

tion into an animal. Many such carrier molecules are known in the art and include, but are not limited to, keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) and cytochrome c. Methods for cross-linking are also well known in the art and include the use of bifunctional cross-linking reagents, such as glutaraldehyde.

5 Such antibodies, as well as antibodies to intact natural phospholipase A₂ or to recombinant phospholipase A₂, are useful in humans and other mammals as anti-inflammatory therapeutics and disease modifying agents in diseases where pathogenic phospholipase A₂ has been implicated. The methods of treatment and their dosage levels and requirements 10 are well recognized in the art and they may be chosen by those of skill in the art from available methods and techniques. For example, the antibodies may be combined with a pharmaceutically acceptable adjuvant for administration to a patient in an amount effective to provide anti-inflammatory effects and 15 accordingly to lessen the severity and course of symptoms. The dosage and treatment regimens will depend upon factors such as the patient's health status, the severity and course of symptoms and the 20 judgment of the treating physician.

25 Diseases which may be treated by compositions characterized by antibodies to phospholipase A₂ include, viral and bacterial infections, skin and connective tissue diseases, such as psoriasis, 30 gastrointestinal disorders, such as pancreatitis and ulcers, ischemias, myocardial infarction, atherosclerosis, pulmonary dysfunctions, such as asthma, acute respiratory distress syndrome and alveolar proteinosis, septic shock, thrombosis, multiple 35 sclerosis, demyelinating diseases and rheumatoid arthritis. These antibodies may also be employed as diagnostics in determining phospholipase A₂ levels

in tissues, body fluids, inflammatory cells and other cells using any conventional immunoassay technique. Phospholipase A₂ purified by the process of this invention, or recombinantly made, may be 5 used in drug screening programs designed to search for inhibitors that can be used as anti-inflammatory, anti-arthritis and anti-thrombotic agents.

The present invention also relates to DNA sequences which encode all or a portion of the amino 10 terminal 19 amino acids of human inflammatory phospholipase A₂. Such DNA sequences are preferably synthesized as a combination of oligonucleotides to account for the degeneracy of the genetic code. These DNA sequences, individually or in combination, 15 are useful as probes to permit the isolation and selection of DNA sequences coding for intact phospholipase A₂ and phospholipase A₂-like polypeptides from various DNA and cDNA libraries, the synthesis of which is well-known in the art. Such DNA sequences, defined herein as "PLA₂ inserts", recombinant molecules including them and unicellular 20 hosts transformed with them may be employed to produce large amounts of phospholipase A₂, substantially free from other proteins of human origin. Such 25 libraries include chromosomal gene banks and cDNA or DNA libraries prepared from tissue or cell lines that are demonstrated to produce phospholipase A₂. These cell lines, as well as techniques for constructing DNA and cDNA libraries, are well known in the 30 art.

The DNA sequences of the present invention either intact or portions thereof, are also useful to probe phospholipase A₂ mRNA levels in inflammatory cells (e.g., neutrophils, monocytes, 35 lymphocytes) and many other cells (e.g., synoviocytes, endothelial cells, smooth muscle cells).

For the purpose of this application, phospholipase A₂-like polypeptides are defined as poly-

peptides which 1) are recognized by antibodies to native phospholipase A₂ in any standard immunoassay, or 2) will elicit antibodies which recognize native phospholipase A₂ in any standard immunoassay, or 5 3) demonstrate phospholipase A₂ enzymatic activity.

It should be understood that a variety of cloning and selection techniques might theoretically be useful in locating and identifying DNA or cDNA sequences of this invention that encode phospholipase A₂ other than the hybridization of oligonucleotides to genomic clones illustrated in the following examples. [See e.g., T. Maniatis et al., "Molecular Cloning - A Laboratory Manual", Cold Spring Harbor (1982).] If these alternate techniques do not yield 10 a DNA or cDNA clone which encodes the entire phospholipase A₂ polypeptide, the selected DNA sequences 15 may themselves be used as probes to select other DNA sequences coding full-length phospholipase A₂.

Partial or full-length DNA or cDNA sequences 20 may be used in appropriate recombinant DNA molecules to transform appropriate eukaryotic and prokaryotic hosts for the production of the phospholipase A₂ and phospholipase A₂-like polypeptides encoded by them.

The DNA sequences and recombinant DNA 25 molecules of the present invention may be expressed using a wide variety of host/vector combinations. For example, useful vectors may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences, such as various known derivatives of SV40 30 and known bacterial plasmids, e.g., plasmids from E.coli including col E1, pCRL, pBR322, pMB9 and RP4, phage DNAs, e.g., the numerous derivatives of λ phage, e.g., NM 989, and other DNA phages, e.g., M13 and other filamentous single-stranded DNA phages, 35 vectors useful in yeasts, such as the 2 μ plasmid, vectors useful in animal cells, such as those containing SV-40, adenovirus and retrovirus derived DNA

sequences (e.g., BG368 and BG341) and vectors derived from combinations of plasmids and phage DNAs, such as plasmids which have been modified to employ phage DNA or other derivatives thereof.

5 Such expression vectors are also characterized by at least one expression control sequence that may be operatively linked to the phospholipase A₂ DNA sequence inserted in the vector in order to control and to regulate the expression of that

10 cloned DNA sequence. Examples of useful expression control sequences are the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage λ , the control region of fd coat protein, the glycolytic promoters of yeast, e.g., the promoter for 3-phosphoglycerate kinase, the promoters of yeast acid phosphatase, e.g., Pho5, the promoters of the yeast α -mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g., the early and late

15 promoters of SV40, the major late promoter of adenovirus, and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells and their viruses or combinations thereof.

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Among such useful expression vectors are

25 vectors that enable the expression of the cloned phospholipase A₂-related DNA sequences in eukaryotic hosts, such as animal and human cells [e.g., P. J. Southern and P. Berg, J. Mol. Appl. Genet., 1, pp. 327-41 (1982); S. Subramani et al., Mol. Cell. Biol., 1, pp. 854-64 (1981); R. J. Kaufmann and P. A. Sharp, "Amplification And Expression Of Sequences Cotransfected with A Modular Dihydrofolate Reductase Complementary DNA Gene", J. Mol Biol., 159, pp. 601-21 (1982); R. J. Kaufmann and P. A.

30 Sharp, Mol. Cell. Biol., 159, pp. 601-64 (1982); S. I. Scahill et al., "Expression And Characterization Of The Product Of A Human Immune Interferon

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-18-

DNA Gene In Chinese Hamster Ovary Cells", Proc.
Natl. Acad. Sci. U.S.A., 80, pp. 4654-59 (1983);
G. Urlaur and L. A. Chasin, Proc. Natl. Acad. Sci.
USA, 77, pp. 4216-20 (1980)].

5 Furthermore, within each specific expression vector, various sites may be selected for insertion of the PLA₂ inserts of this invention. These sites are usually designated by the restriction endonuclease which cuts them. They are well recognized by those
10 of skill in the art. It is of course to be understood that an expression vector useful in this invention need not have a restriction endonuclease site for insertion of the chosen DNA fragment. Instead, the vector could be joined to the fragment by alternative means. The expression vector, and in particular the site chosen therein for insertion of a selected DNA fragment and its operative linking therein to an expression control sequence, is determined by a variety of factors, e.g., number of sites susceptible to a
15 particular restriction enzyme, size of the protein to be expressed, susceptibility of the desired protein to proteolytic degradation by host cell enzymes, contamination or binding of the protein to be expressed by host cell proteins difficult to remove
20 during purification, expression characteristics, such as the location of start and stop codons relative to the vector sequences, and other factors recognized by those of skill in the art. The choice of a vector and an insertion site for a DNA sequence
25 is determined by a balance of these factors, not all selections being equally effective for a given case.

30 Useful expression hosts may include well known eukaryotic and prokaryotic hosts, such as strains of E.coli, such as E.coli SG-936, E.coli HB 101, E.coli W3110, E.coli X1776, E.coli X2282, E.coli MC1061, E.coli DHI, and E.coli MRCl, Pseudomonas, Bacillus, such as Bacillus subtilis,

Streptomyces, yeasts and other fungi, animal cells, such as COS cells and CHO cells, human cells, insect cells and plant cells in tissue culture.

Of course, not all host/expression vector combinations function with equal efficiency in expressing the DNA sequences of this invention or in producing the phospholipase A_2 -like polypeptides. However, a particular selection of a host-expression vector combination may be made by those of skill in the art after due consideration of the principles set forth herein without departing from the scope of this invention. For example, the selection should be based on a balancing of a number of factors. These include, for example, compatibility of the host and vector, toxicity of the proteins encoded by the DNA sequence to the host, ease of recovery of the desired protein, expression characteristics of the DNA sequences and the expression control sequences operatively linked to them, biosafety, costs and the folding, form or any other necessary post-expression modifications of the desired protein.

Alternatively, if the isolated genomic clone contains phospholipase A_2 expression control sequences, such as promoters, ribosome binding sites, and polyadenylation signals in addition to the phospholipase A_2 coding sequence, expression vectors may be unnecessary. In this case, the genomic clone alone may be used to transfect eukaryotic hosts, which, in turn can express phospholipase A_2 . Such hosts include cells well-known in the art, such as mouse L-cells or CHO cells.

Thus, the present invention provides three different methods in which to produce recombinant phospholipase A_2 -like polypeptides. These methods include heterologous promoter-mediated expression of phospholipase A_2 cDNAs, heterologous promoter-regulated expression of phospholipase A_2 genomic DNA, and native

-20-

promoter-mediated expression of phospholipase A₂ genomic DNA. The latter two methods are only applicable to eukaryotic cells that are able to perform proper splicing out of introns.

5 It should be understood that in addition to the DNA and cDNA sequences described herein, the present invention also relates to DNA sequences which hybridize to the foregoing DNA sequences, as well as DNA sequences which, due to the degeneracy 10 of the genetic code, code on expression for human phospholipase A₂-like polypeptides coded for on expression by the foregoing DNA or cDNA sequences.

In order that our invention herein described 15 may be more fully understood, the following examples are set forth. It should be understood that these examples are for illustrative purposes only and should not be construed as limiting this invention in any way to the specific embodiments recited therein.

EXAMPLE 1

20 Purification Of Phospholipase A₂ From Human Platelets.

According to one embodiment of this invention, we purified phospholipase A₂ from human platelets as follows:

25 A. Preparation Of Human Platelets

We centrifuged a preparation of normal human platelet concentrates (obtained from the Red Cross; within 5 days of phlebotomy) at 200xg in a Sorvall RT6000B centrifuge for 10 min at room temperature to remove any residual red cells. The 30 supernatant was made 1 mM in EGTA and the platelets pelleted by centrifugation at 2,500xg for 15 min at 4°C. We washed the pellets with 120 mM NaCl, 2 mM EGTA, 30 mM Tris-HCl, pH 7.4 and then resuspended 35 the platelets in that buffer at a protein concentra-

tion of 10 mg/ml. These preparations were quick frozen in a dry ice-acetone bath and stored in 10 ml aliquots at -70°C before further use.

B. Acid-Extraction Of Human Platelets

5 We thawed 750 ml of the above-prepared platelets (7510 mg protein) and sonicated them in three batches at 4°C with a probe sonicator using a 3-4 inch standard horn (Model W-225; Heat Systems-Ultrasonics). Each batch received six 15 second
10 pulses at an output setting of 6 with a 45 second interval between each pulse. The sonicates were pooled and mixed with an equal volume of ice-cold 0.36 N sulfuric acid and let stand at 4°C for 60 min. Precipitated material was separated by centrifugation
15 at 10,000xg for 30 min at 4°C. We collected the supernatant and reextracted the pellets in a total of 500 ml of 0.18 N sulfuric acid containing 150 mM NaCl on ice for 60 min. The remaining insoluble material was pelleted by centrifugation as
20 above. The supernatants from both extracts were pooled (2000 ml) and dialyzed overnight against 3 x 16 liters of 200 mM NaCl, 50 mM sodium acetate, pH 4.5 using Spectra/Por membranes (3500 dalton molecular weight cutoff). We centrifuged the
25 dialyzed platelet preparation at 15,000xg for 40 min at 4°C to remove any precipitated material.

C. Partial Purification Of Human Platelet PLA₂ Activity By Cation Exchange Chromatography

30 We applied the supernatant (718 mg protein) to a 1.6 x 27 cm Fast S Sepharose (Pharmacia) column that had been pre-equilibrated with 200 mM NaCl, 50 mM sodium acetate, pH 4.5 at a flow rate of 90 ml/h. After washing the column with 150 ml of the same
35 buffer, we developed it with 550 ml of a linear salt gradient from 200 mM - 2 M NaCl in 50 mM sodium ace-

-22-

tate, pH 4.5. Fractions (5 ml) were collected and assayed for absorbance at 280 nm and for phospholipase A₂ activity. Figure 1 shows that the phospholipase A₂ eluted with approximately 1 M NaCl at fractions 5 74-82. We pooled these fractions and concentrated them to 0.8 ml using an Amicon ultrafiltration stirred cell with a YM 5 membrane.

10 D. Partial Purification Of Human Platelet PLA₂ Activity By Gel Filtration

We then chromatographed the concentrated peak fractions from the cation exchange column on a Sephadex G-50 superfine (Pharmacia) column (1 x 48 cm) which had been pre-equilibrated in 500 mM NaCl, 15 50 mM sodium acetate, pH 4.5. We collected 0.5 ml fractions at a flow rate of 2 ml/h. Fractions were assayed for absorbance at 280 nm and for phospholipase A₂ activity. Figure 2 demonstrates that enzymatic activity eluted in fractions 45-56, with 20 an apparent molecular weight of 13,000 daltons.

E. Purification Of Human Platelet PLA₂ By Reverse-Phase HPLC

The pooled peak fractions from the gel filtration column (6 ml; 100 µg protein) were further purified on a C4 reverse-phase HPLC column 25 (Vydac; 0.46 x 25 cm) that was equilibrated at 29°C with 0.1% trifluoroacetic acid (TFA). The reverse-phase column was developed at a flow rate of 1 ml/min with a 45 minute gradient (0-75% acetonitrile in 0.1% TFA), collecting 0.5 ml fractions. The column eluate was monitored for absorbance at 214 nm (AFU 0.2) and 30 280 nm (AFU 0.05). An aliquot of each fraction was diluted into 500 mM NaCl, 50 mM acetate, pH 4.5 containing 1 mg/ml bovine serum albumin (Sigma) and 35 assayed for activity. Figure 3, panel B, demonstrates that approximately 35% of the applied phospholipase

-23-

A_2 activity was recovered in a single peak contained in fraction 45. Figure 3, panel A, indicates the activity eluted at about 32% acetonitrile. We added 1 μ l of 5% SDS to this fraction and dried the sample 5 in a Speed-Vac concentrator (Savant).

Figure 4 depicts, in tabular form, the entire purification process. The final yield of phospholipase A_2 from platelets was 34% and the protein was purified over 1,100,000-fold over the 10 starting material. After extraction and dialysis, the total phospholipase A_2 activity increased 63-fold over that observed in the sonicate and was assumed to be 100%. The purification-fold was estimated assuming 100% recovery of enzymatic activity 15 during these steps.

F. SDS-PAGE/Electroblotting Onto PVDF Membrane Of Human Platelet PLA₂

We dissolved the PLA₂ protein in 25 μ l of electrophoresis sample buffer, incubated the sample 20 for 10 min at 60°C, loaded it onto a minigel (5 x 7 cm) containing a 16% SDS-polyacrylamide gel and a 5% stacking gel and electrophoresed at 20 mA constant current for 120 min (U. K. Laemmli, supra). Following electrophoresis, we soaked the gel in trans- 25 fer buffer (10 mM 3-[cyclohexylamino]- 1-propanesulfonic acid, 10% methanol, 0.05% SDS, pH 11.25) for 5 min and electroblotted onto a PVDF membrane (Immobilon; 0.45 μ m pore size, Millipore) for 1 h at 150 mA (P. Matsudaira, "Sequence from Picomole Quantities of 30 Proteins Electroblotted Onto Polyvinylidene Difluoride Membranes", J. Biol. Chem., 262, pp. 10035-38 (1987)). We visualized the proteins by staining the membrane with Coomassie Blue R-250 (Figure 5). We then rinsed the membrane extensively with deionized water, dried 35 it and stored it at -20°C.

-24-

EXAMPLE 2

Purification Of Phospholipase A₂
From Human Synovial Fluid

According to one embodiment of this invention, we purified phospholipase A₂ from human synovial fluid as follows:

A. Preparation Of Human
Rheumatoid Synovial Fluid

Synovial fluid was aspirated from patients diagnosed with classical rheumatoid arthritis, as defined by American Rheumatism Association criteria. We removed cells and debris from synovial fluids by centrifugation at 4°C for 20 min at 3,000xg in a Sorvall RC3B centrifuge. The synovial fluids were stored at -70°C before further use.

B. Acid Extraction Of
Rheumatoid Synovial Fluid

We thawed the synovial fluids, pooled them to yield 50 ml (1932 mg protein) and mixed them with an equal volume of 0.36 N sulfuric acid. To this we added 100 ml of 0.18 N sulfuric acid containing 150 mM NaCl and incubated the mixture on ice for 60 min. The mixture was then dialyzed overnight against 2 x 4 liters of 200 mM NaCl, 50 mM Na acetate, pH 4.5 (Spectra-Por membranes; 3500 dalton cutoff). We then removed precipitated material by centrifuging at 15,000xg for 40 min at 4°C.

C. Partial Purification Of Human
Synovial Fluid PLA₂ Activity
By Cation Exchange Chromatography

We applied the supernatant (1582 mg protein) to a 1.6 x 27 cm Fast S Sepharose (Pharmacia) column that had been pre-equilibrated with 200 mM NaCl, 50 mM sodium acetate, pH 4.5 at a flow rate of 90 ml/h. After we washed the column with 150 ml of

the same buffer, we developed with 550 ml of a linear salt gradient from 200 mM - 2 M NaCl in 50 mM Na acetate pH 4.5. Fractions (5 ml) were collected and assayed for protein content, by measuring absorbance at 280 nm and for phospholipase A₂ activity. Figure 6 shows that the protein eluted from the column with approximately 1 M NaCl at fractions 84-98. We pooled these fractions and concentrated them to 0.8 ml using an Amicon ultrafiltration stirred cell with a YM 5 membrane.

10

D. Purification Of Human Rheumatoid Synovial Fluid PLA₂ Activity By Gel Filtration

15 We then chromatographed the concentrated peak fractions from the cation exchange column (2.5 mg protein) on a Sephadex G-50 superfine (Pharmacia) column (1 x 48 cm) which had been pre-equilibrated in 500 mM NaCl, 50 mM Na acetate, pH 4.5. We collected 0.5 ml fractions at a flow rate of 2 ml/h.

20 Fractions were assayed for absorbance at 280 nm and for phospholipase A₂ activity. Figure 7 demonstrates that enzymatic activity eluted in fractions 45-56, with an apparent molecular weight of 13,000 daltons.

25

E. Purification Of Human Rheumatoid Synovial Fluid PLA₂ By Reverse Phase HPLC

30 The pooled peak fractions from the gel filtration column (6 ml; 100 µg protein) were further purified on a C4 reverse-phase HPLC column (Vydac; 0.46 x 25 cm) that was equilibrated at 29°C with 0.1% trifluoroacetic acid (TFA). The column was then developed at a flow rate of 1 ml/min with a 45 minute gradient (0-75% acetonitrile in 0.1% TFA), collecting 0.5 ml fractions. The column eluate was 35 monitored at 214 nm (AUF 0.2) and 280 nm (AUF 0.05). An aliquot of each fraction was diluted into 500 mM NaCl, 50 mM acetate, pH 4.5 buffer containing 1 mg/ml

-26-

bovine serum albumin and assayed for phospholipase A₂ activity. Figure 8, panel B, demonstrates that the phospholipase A₂ activity was recovered in a single peak contained in fractions 48 and 49.

5 Figure 8, panel A, indicates the activity eluted at about 30% acetonitrile. We added 1 μ l of 5% SDS to these fractions and dried the samples in a Speed-Vac concentrator (Savant).

10 Figure 9 demonstrates, in tabular form, the entire purification process. The final yield of phospholipase A₂ from rheumatoid synovial fluid was 57% and the protein was purified over 100,000-fold over the starting material.

15 F. SDS PAGE/Electroblotting Onto
PVDF Membrane Of Human
Rheumatoid Synovial Fluid PLA₂

We dissolved the PLA₂ protein in 25 μ l of electrophoresis sample buffer, incubated the sample for 10 min at 60°C, loaded it onto a minigel 20 (5 x 7cm) containing a 16% SDS-polyacrylamide gel and a 5% stacking gel and electrophoresed at 20 mA constant current for 120 min. Following electrophoresis, we soaked the gel in transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% 25 methanol, 0.05% SDS, pH 11.25) for 5 min and electroblotted onto a PVDF membrane (Immobilon; 0.45 μ m pore size, Millipore) for 1 h at 150 mA. We visualized the proteins by staining the membrane with Coomassie Blue R-250 (Figure 10). We then rinsed 30 the membrane extensively with deionized water, dried it and stored it at -20°C.

EXAMPLE 3

Preparation Of Substrate And
Assay of Phospholipase A₂ Activity

35 We prepared the substrate, ³H-oleic acid-labelled E.coli, as follows: We grew an overnight

culture of E.coli in 1% bactotryptone, 0.5% NaCl, diluted it 1:20 into fresh broth and allowed the cells to regrow, monitoring cell growth with a Klett-Summerson colorimeter, until the absorbance 5 reached 40. We then added 1/100th of a volume of 10% Brij 35 (Sigma Chemicals) and 1/200th of a volume of ³H-oleic acid (9,10-³H-[N]-oleic acid, New England Nuclear) at 10 mCi/ml to the culture. After 5h of growth, we autoclaved the culture and stored the 10 bacteria overnight at 4°C. We then pelleted the bacteria by centrifugation (16,000 rpm, 30 min, 4°C, SS34 rotor), combined the loose pellets and washed 4 times in 0.7 M Tris-HCl, 10 mM CaCl₂, 0.1% BSA, pH 8.0, until radioactivity in the supernatant was low. 15 The bacteria were stored in this buffer containing 0.2% Na azide at 4°C. We then prepared, for example, a 400 ml culture labeled with 20 mCi of ³H-oleic acid. This yielded about 7×10^8 counts per minute or about 10% of the input counts in labeled bacteria. 20 Prior to use in an assay, we washed aliquots of cells for 30 min on ice in 200 mM Tris-HCl, 12 mM EDTA, pH 8.0, followed by 25 mM Tris-HCl, pH 8.0. A typical assay used, for each point, 100,000 cpm, which was added in a volume of 25 μ l.

25 We performed a typical phospholipase A₂ assay as follows: Samples to be assayed for phospholipase A₂ activity (20 μ l) were mixed with 25 μ l of autoclaved [³H]-oleic acid-labeled E.coli as substrate and brought to a total volume of 200 μ l 30 with 0.1 M Tris-HCl, pH 9, containing 10 mM CaCl₂, 1 mM 2-mercaptoethanol and 1 mg/ml BSA. The reaction was incubated at 37°C for 15 min and stopped by the addition of 100 μ l of 2N HCl, followed by 100 μ l of delipidated BSA. Samples were vortexed and incubated 35 on ice for 30 min. The samples were spun in an Eppendorf microcentrifuge for 5 min at 10,000xg and 250 μ l of the supernatants containing released

-28-

[³H]oleic acid were counted for radioactivity after mixing with 4 ml of scintillation fluid compatible with aqueous solutions (Fisher). A unit of activity for human rheumatoid synovial fluid phospholipase A₂ was defined as the amount of protein necessary to release 1 x 10⁹ cpm of [³H]-oleate in 15 min at 37°C. A unit of activity for human platelet phospholipase A₂ was defined as the amount of protein necessary to release 1 x 10⁶ cpm of [³H]-oleate in 15 min at 37°C.

EXAMPLE 4

Amino Acid Sequence Analysis

The major band visualized on the PVDF membrane following electrophoresis and electroblotting was excised and subjected to automated Edman degradation (P. Matsudaira, J. Biol. Chem., 262, pp. 10035-38 (1987)) using an Applied Bio-systems 470A gas phase protein sequencer equipped with a model 900A data system (R. M. Hewick et al., 20 "A Gas-Liquid Solid Phase Peptide and Protein Sequenator", J. Biol. Chem., 256, pp. 7990-97 (1981)). The resulting phenylthiohydantoin amino acids were analyzed on-line using an Applied Bio-systems 120A PTH amino acid analyzer equipped with a 25 PTH-C18 column (2.1 x 220 mm). The amino terminal 19 amino acids were determined for both platelet and synovial fluid phospholipase A₂. Both proteins had the identical amino terminal sequence of H₂N-Asn-Leu-Val-Asn-Phe-His-Arg-Met-Ile-Lys-Leu-Thr-Thr-30 Gly-Lys-Glu-Ala-Ala-Leu.

EXAMPLE 5

Synthesis Of Human Inflammatory Phospholipase A₂ Polypeptides

We synthesized a 16 amino acid polypeptide, 35 Leu-Val-Asn-Phe-His-Arg-Met-Ile-Lys-Leu-Thr-Thr-

Gly-Lys-Glu-Ala, corresponding to amino acids 2-17 of the above-sequenced phospholipases A₂. We prepared the polypeptide by solid-phase synthesis employing an Applied Biosystems 430A Peptide

5 Synthesizer, using the procedures of R. B. Merrifield, "Solid Phase Peptide Synthesis. I. Synthesis of a Tetrapeptide", J. Amer. Chem. Soc., 85, pp. 2149-54 (1963)

The polypeptide was coupled to keyhole 10 limpet hemocyanin with glutaraldehyde before being used to immunize rabbits. For primary injection, 1.5 mg of the polypeptide-KLH complex was emulsified with Freund's complete adjuvant and administered intramuscularly. For subsequent injections, 0.75 mg 15 of the polypeptide-KLH complex was emulsified with Freund's incomplete adjuvant and administered intramuscularly. Animals were bled every 2 weeks and sera assayed for anti-human inflammatory phospholipase A₂ titer by ELISA.

20 Other phospholipase A₂-like polypeptides, as well as mature phospholipase A₂ may be similarly synthesized and used to raise anti-human inflammatory phospholipase A₂ antibodies. Additionally, phospholipase A₂-like polypeptides of sufficient 25 size and immunogenicity may be used directly to elicit antibodies to phospholipase A₂ without coupling to KLH.

EXAMPLE 6

30 Synthesis Of Human Inflammatory Phospholipase A₂ Oligonucleotides

Unless otherwise specified, all molecular biological techniques are described in T. Maniatis et al., Molecular Cloning, Cold Spring Harbor, New York (1982).

35 We synthesized a number of oligonucleotides based on three different hexapeptides of the deduced

-30-

amino acid sequence of human inflammatory phospholipase A₂. The three hexapeptides were: Asn-Phe-His-Arg-Met-Ile, Met-Ile-Lys-Leu-Thr-Thr, and Thr-Thr-Gly-Lys-Glu-Ala. More than one nucleotide was introduced at various positions during the synthesis of these oligonucleotides, to account for the degeneracy of the genetic code. Thus, the product of any single oligonucleotide synthesis was actually a mixture of oligonucleotides, all of which potentially coded for the corresponding hexapeptide. For the purposes of the present specification and claims, the following code is used to designate nucleotides:

A- adenine
N- adenine, thymidine, guanidine, or cytidine
15 T- thymidine
Pu- adenine or guanidine
G- guanidine
Py- thymidine or cytidine
C- cytidine
20 Z- adenine, guanidine or thymidine

The following oligonucleotides based on the hexapeptide Asn-Phe-His-Arg-Met-Ile were synthesized:

25 PLA₂-06: 5' ATC ATPu CGPu TGpu AAPu TT 3'
PLA₂-07: 5' ATC ATPy CGPu TGpu AAPu TT 3'
PLA₂-08: 5' ATC ATPy CTPu TGpu AAPu TT 3'

The following oligonucleotides based on the hexapeptide Met-Ile-Lys-Leu-Thr-Thr were synthesized:

30 PLA₂-09: 5' GTN GTPy AAPy TTZ ATC AT 3'
PLA₂-10: 5' GTN GTPu AGPy TTZ ATC AT 3'
PLA₂-11: 5' GTN GTPy AGPy TTZ ATC AT 3'

The following oligonucleotides based on the hexapeptide Thr-Thr-Gly-Lys-Glu-Ala were synthesized:

35 PLA₂-12: 5' GCPy TCPy TTPu CCPu GTPu GT 3'
PLA₂-13: 5' GCPy TCPy TTPy CCPy GTPy GT 3'

All of the oligonucleotides were synthesized on an Applied Biosystems 380A automated DNA

synthesizer, using the procedure described by L. J. McBride and M. H. Caruthers, "The Synthesis of Oligodeoxypyrimidines on a Polymer Support", Tetrahedron Letters, 24, pp. 245-48 (1983).

5

EXAMPLE 7Construction Of A Human Genomic Library

High molecular weight DNA was isolated from monolayer cultures of a mutant fibroblast cell line which contains 5 copies of the X chromosome (Human Genetic Mutant Cell Repository, Camden, New Jersey; repository number GM5009) using standard techniques. We then partially digested the DNA with restriction enzyme Sau3A and dephosphorylated the fragments with bacterial alkaline phosphatase. Subsequently, we ligated the fragments to BamHI-digested λEMBL3 DNA (A.-M. Frischauf et al, "Lambda Replacement Vectors Carrying Polylinker Sequences", J. Mol. Biol., 170, 827-42 (1983)) and packaged the bacteriophage genomes using a two-extract kit according to manufacturer's directions (Amersham Corp.). Recombinant bacteriophage were selected by plating on E.coli MP801 cells (a gift of Dr. Mark Pasek, Biogen Inc., Cambridge, MA), a P2 lysogen of SG4119.

25

EXAMPLE 8Screening A Human Genomic Library
For Inflammatory Phospholipase A₂ Sequences

We initially screened the GM5009 human genomic DNA EMBL3 phage library for phospholipase A₂ sequences with a combination of oligonucleotides PLA₂-06, PLA₂-07 and PLA₂-08 by plaque hybridization screening (S.L.C. Woo, "A Sensitive and Rapid Technique for Recombinant Phage Screening", Meth. Enzymol., 68, pp. 389-96 (1979)).

-32-

We grew a culture of E.coli LE392 cells in L-broth plus 0.2% maltose overnight at 37°C. We then pelleted the cells by centrifugation and resuspended the cell pellet in an equal volume of SM buffer. For each plate, we pre-adsorbed 0.9 ml of cells with 2×10^5 phage particles at room temperature for 15 min. We then added 50 ml of L-broth plus 10 mM MgSO₄ and 0.7% agarose (melted and held at 55°C) and plated the mixture onto ten LB-MgSO₄ Nunc plates (25 cm x 25 cm). The plates were incubated at 37°C for 8 h or until the plaques were just nearly touching. The plates were then chilled at 4°C to allow the agarose to harden.

We presoaked Genescreen Plus filters (New England Nuclear) in a 1:10 dilution of an overnight culture of LE392 cells for 10 min at room temperature in order to coat each filter with bacteria. After air drying, the filters were contacted with the plates containing the recombinant plaques for 5 min. The filters were removed and placed phage-side up onto LB plus 10 mM MgSO₄ plates. A second replica lift was made from each plate by the same procedure. We then incubated all filters at 37°C for 5 h. After incubation, we removed the filters from the plates and placed them in a pool of 0.5 N NaOH, 1.5 M NaCl, two times, to lyse the phage. The filters were then neutralized in 0.5 M Tris-HCl pH 7.0, 1.5 M NaCl and scrubbed free of cell debris.

We ³²P-labeled a combination of oligonucleotides PLA₂-06, PLA₂-07 and PLA₂-08 with polynucleotide kinase and high specific activity ³²P-ATP using standard techniques. We pre-hybridized the filters in plaque screen buffer for 1 h and then hybridized to the above labeled probes at 45°C for 15 h in plaque screen buffer containing 10% dextran sulfate and 100 µg/ml yeast tRNA according to the

manufacturer's specifications for plaque screen membranes (New England Nuclear).

The filters were then washed two times in plaque screen buffer at 45°C, followed by two washes 5 in 3.2 M tetramethylammonium chloride "(TMACl)", 1% SDS at 45°C (P. E. Devlin et al, "Southern Analysis of Genomic DNA With Unique and Degenerate Oligonucleotide Probes: A Method for Reducing Probe Degeneracy", DNA, (in press) (1988)). Positive phage were 10 detected by autoradiography. We selected 64 positive plaques by this technique. Agarose plugs containing the positive plaques were removed from the master plate, transferred into SM buffer and 18 of these were rescreened at lower density using the 15 same technique. For rescreening, we used nitrocellulose filters and included a final wash in 1 M ammonium acetate following neutralization in Tris-HCl-NaCl. The filters were baked at 80°C for 2 h following this wash.

20 Ten of the rescreened clones remained positive and at least one clone also hybridized to a combination of probes PLA₂-09, PLA₂-10 and PLA₂-11 which had been ³²P-labeled with polynucleotide kinase. This clone is referred to as PLA₂ 8.5 EMBL3.

25 We isolated DNA from clone PLA₂ 8.5 EMBL3 and determined the insert to be 16 kilobases (kb) upon restriction enzyme digestion analysis. Using the Southern Blot technique (E. M. Southern et al., "Detection of Specific Sequences Among DNA Fragments Separated by Gel Electrophoresis", J. Mol. Biol., 30 98, pp. 503-18 (1975)) we determined that the sets of probes PLA₂-06 to PLA₂-08 and PLA₂-09 to PLA₂-11 all hybridized to a single 6.2 kb HindIII fragment of PLA₂ 8.5 EMBL3 ("the 6.2 kb PLA₂ insert"). This 35 fragment was isolated following digestion of the clone with HindIII and SalI and subsequent electrophoresis in a 0.8% low-melting agarose gel. The

-34-

actual 6.2 kb HindIII fragment isolated did not contain a SalI site, but this digestion was necessary to eliminate a similarly-sized HindIII fragment of PLA₂ 8.5 EMBL3. This latter fragment contains a 5 SalI site and thus the double digestion eliminates the possibility of contamination.

The fragment was cloned into HindIII-digested pNN01 that had been treated with calf intestinal alkaline phosphatase. We constructed the 10 sequencing plasmid pNN01 by removing the synthetic polylinker from the commercially available plasmid pUC8 (Pharmacia PL Biochemicals) by restriction digestion and replacing it with a new synthetic segment. The 2.5 kb backbone common to the pUC plasmids, 15 which provides an origin of replication and confers ampicillin resistance, remained unchanged. The novel synthetic portion of pNN01 is shown in Figure 11A.

For ligation, we mixed 10 ng of digested vector with 40 ng of the 6.2 kb PLA₂ insert in 100 20 μ l of T4 DNA ligase buffer containing 400 units of T4 ligase. Ligation was achieved by incubation at room temperature for 5 h. We used 20 μ l of the ligation mixture to transform 0.2 ml of competent E.coli MC1061 cells. Transformants were grown on 25 LB + ampicillin plates at 37°C overnight. We isolated plasmid DNA from 12 potential transformants using the alkaline miniprep procedure (T. Maniatis et al., supra) and determined that one transformant, PSQ 130, contained the 6.2 kb PLA₂ insert (Figure 11B). 30 The insert was then sequenced using the method of Maxam and Gilbert (A. M. Maxam and W. Gilbert, "A New Method for Sequencing DNA", Proc. Natl. Acad. Sci. USA, 74, pp. 560-64 (1977)). A BamHI-NcoI fragment of the 6.2 kb PLA₂ insert, which hybridized to 35 the PLA₂ oligonucleotide probes of this invention, had the following nucleotide sequence:

C CAT GGG AAT TTG GTG AAT TTC CAC AGA ATG
ATC AAG TTG ACG ACA GGA AAG GAA GCC GCA CTC AGT TAT
GGC TTC TAC GGC TGC CAC TGT GGC GTG GGT GGC AGA GGA
TCC.

5 This sequence encodes the polypeptide:

His Gly Asn Leu Val Asn Phe His Arg Met
Ile Lys Leu Thr Thr Gly Lys Glu Ala Ala Leu Ser Tyr
Gly Phe Tyr Gly Cys His Cys Gly Val Gly Gly Arg Gly
Ser.

10 The underscored 19 amino acids of this polypeptide correspond exactly with the protein sequence information obtained from purified human platelet and rheumatoid synovial fluid phospholipase A₂, confirming that we had isolated at least part of 15 the genomic clone. The additional 3' end region of this region encodes amino acids that correspond to conserved sequences in other phospholipases A₂ that have been sequenced.

20 The entire DNA sequence of the 6.2 kb PLA₂ insert as well as the amino acid sequence coded for in the exons is shown in Figure 12. Potential intron splice sites are indicated by arrowheads. The coding sequence for mature phospholipase A₂ begins at nucleotide 2722 (arrow, Figure 12) and is contained within 25 exon 2. Exon 2 begins at nucleotide 2702 and encodes 6 in-frame amino acids preceding the amino terminal asparagine residue of mature phospholipase A₂. We believe that these 6 amino acids encode the carboxy 30 terminal 6 amino acids of the phospholipase A₂ signal sequence.

35 An open reading frame of 14 amino acids beginning with a methionine residue and having characteristic properties of a signal sequence is located between nucleotides 2453 and 2492. This nucleotide sequence also terminates with a characteristic GT splice site at nucleotides 2493-2494. It is unlikely that this represents the N-terminal portion of the

-36-

in vivo signal, because no promoter-like sequences are found within the 100 nucleotides located 5' to this region.

5 Exons 3 and 4, which encode the remainder of phospholipase A₂ and an in-frame stop codon, are located at nucleotides 3105-3211 and 5383-5523, respectively. The putative polyadenylation signal, AATAAA, is located at nucleotides 5771-5776 (under-scored in Figure 12).

10 The mature PLA₂ polypeptide coded for by exons 2, 3 and 4 consists of 124 amino acids and has the formula: NLVN FHRMIK LTTG KEEA ALS YGFY GCHCGV GGRG SPK DAT DRCC VTH DCC YKR LEK RGCG TKFL SYK FSN SG SRIT CAK Q DSCR SQL CEC DKAA ATCFAR NK ITTY NKKY Q YY SNK HCR GS TPR C.

15 In the above-cited formula as well as throughout this application the amino acids are represented by single letter codes as follows:

Phe: F	Leu: L	Ile: I	Met: M
Val: V	Ser: S	Pro: P	Thr: T
Ala: A	Tyr: Y	His: H	Gln: Q
Asn: N	Lys: K	Asp: D	Glu: E
Cys: C	Trp: W	Arg: R	Gly: G

20 We believe that the 3 exons of the genomic clone contained within the 6.2 kb PLA₂ EMBL3 8.5
25 Hind III fragment encode phospholipase A₂) based on the following observations. First, the clone encodes the identical N-terminal amino acid sequence identified for the purified native enzyme by protein sequencing (see Example 4). This sequence represents an amphiphilic alpha-helix that is typical for all phospholipases A₂ sequenced to date. It also encodes the highly conserved lipophilic residues within this alpha-helix (e.g., Leu₂, Phe₅ and Ile₉). Furthermore, the clone codes for a cluster of basic
30 amino acids (e.g., Arg₇, Lys₁₁ and Lys₁₅) which is
35

believed to be an important determinant in the interaction of phospholipase A_2 with specific biological targets. Second, the clone encodes a characteristic stretch of amino acids Tyr_{25} -Gly-Cys-X-Cys-Gly-X-Gly-Gly-X-X-Pro₃₇ and Asp₄₉, where X is any amino acid, that are part of the calcium binding loop of phospholipases A_2 . Finally, the clone encodes the characteristic amino acid residues that constitute the active site of all phospholipases A_2 , namely His₄₈, Asp₉₉, Tyr₅₂ and Tyr₇₃.

The phospholipase A_2 amino acid sequence coded for by the 6.2 kb HindIII fragment of genomic DNA clone PLA₂ EMBL3 8.5 also exhibits the placement of half-cysteine residues that is typical for group II phospholipases A_2 . Thus, it contains a cysteine residue at amino acid 50 and has an extension of several amino acid residues at the C-terminus which ends in a half cysteine. A comparison of the amino acid sequence of bovine pancreatic PLA₂, C. atrox venom PLA₂, and the sequence encoded by the 6.2 kb PLA₂ insert as well as a consensus sequence, is depicted in Figure 13.

EXAMPLE 9

25 Construction Of Vectors For The Expression Of Human Inflammatory Phospholipase A_2 In Animal Cells

In order to confirm that the 6.2 kb PLA₂ insert encodes a functional polypeptide and to enable us to obtain mRNA as a source of cDNA, we made several constructs for expression of this phospholipase A_2 sequence in animal cells.

Plasmid PLA₂ 6.2 BG368 3(+) (Figure 14B) was synthesized as follows: PSQ 130 was digested with HindIII and the 6.2 kb PLA₂ insert was isolated by preparative gel electrophoresis through low-melt agarose. The gel slice containing this fragment was excised and stored at 4°C.

-38-

The animal cell expression parent vector, BG368, was constructed as follows: As depicted in Figure 14A, we cut animal cell expression vector BG312 [R. Cate et al., "Isolation Of The Bovine And 5 Human Genes For Mullerian Inhibiting Substance And Expression Of The Human Gene In Animal Cells", Cell, 45, pp. 685-98 (1986)] with EcoRI and BglII to delete one of each of the two EcoRI and the two BglII restriction sites (the EcoRI site at position 0 and 10 the BglII site located at approximately position 99). The resulting plasmid, BG368, retained an EcoRI site in the cloning region and a BglII site after the cloning region. This left a single EcoRI site and a single BglII site in the polylinker for cloning 15 purposes.

More specifically, we deleted one EcoRI site and one BglII site by sequential partial digestion of BG312 with restriction enzymes EcoRI and BglII, respectively. We filled in with Klenow 20 fragment of E. coli polymerase and 4 nucleotides then religated to produce BG368, which contains unique restriction sites for EcoRI and BglII enzymes.

BG368 was linearized by digestion at the unique HindIII site in the polylinker region. BG368 25 contains the SV40 origin of replication and enhancer sequence, the adenovirus major late promoter, a polylinker region containing unique restriction sites for the insertion of DNA sequences for expression, the SV40 3' untranslated region, including the poly- 30 adenylation signal and the 3' splice site. We then treated the linearized vector with calf intestinal alkaline phosphatase to prevent reannealing, phenol extracted it and purified it by preparative gel electrophoresis as above.

35 Slices of low melt agarose containing either the vector or the insert were diluted with H₂O and melted at 65°C for 2-3 minutes. We ligated aliquots,

equivalent to 60 ng and 15 ng, respectively, of 6.2 kb PLA_2 insert and HindIII linearized BG368 vector with 400 units of T4 ligase in a total volume of 40 μ l of 1X T4 ligase buffer overnight at room temperature.

5 We used 20 μ l of this ligation mixture to transform E.coli MC1061 cells. The transformants were grown on LB + ampicillin plates at 37°C overnight. We isolated plasmid DNA from 10 transformants using the alkaline miniprep procedure. By digesting the plasmids with restriction enzymes XhoI and NdeI we determined that three transformants contained the PLA_2 insert. This restriction enzyme analysis also allowed us to determine orientation of this insert with respect to the adenovirus major late promoter in this vector.

10 15 We prepared large scale plasmid preparations of DNA from one transformant containing the + orientation, named PLA_2 6.2 BG368 3(+) and one containing the - orientation, named PLA_2 6.2 BG368 8(-).

We then prepared plasmid PLA_2 3.8 BG341(+) 20 (Figure 14, panel C) as follows: PSQ 130 was digested with NotI to release the entire 6.2 kb PLA_2 insert in addition to the polylinker from pNN01 (Figure 11, panel B). We purified this NotI fragment by gel electrophoresis through 1% agarose in TBE buffer.

25 The fragment was then electroeluted from the gel and recovered by ethanol precipitation. We then digested the NotI fragment with EaqI yielding two fragments approximately 2.4 kb and 3.8 kb in length. We purified the 3.8 kb fragment ("the 3.8 kb PLA_2 insert") 30 by low melt agarose gel electrophoresis as described above. From the nucleotide sequence of the PLA_2 insert we determined that the 3.8 kb PLA_2 insert contains a potential open reading frame encoding an initiating methionine as well as amino acids which 35 are characteristic of signal sequences. This fragment also contains Exons 2, 3, and 4, as well as a

-40-

donor sequence for splicing the amino terminus of the signal sequence to Exon 2.

Plasmid BG341 (Figure 14D) was also derived from BG312 (R. Cate et al., Cell, 45, pp. 685-98 (1986)). After partial digestion of BG312 with BglII to linearize it at the BglII site located at approximately position 99, we filled in the BglII site with Klenow fragment of E.coli DNA polymerase and deoxyribonucleotides then religated the vector. We linearized the vector by partial digestion with BamHI at the site following the SV40 poly A region, filled in the site with Klenow and deoxyribonucleotides and religated the vector. The resultant vector with unique BglII and BamHI sites was linearized with SmaI and a linker having the sequence 5'GC GGCC CGCGCTCGAGCTCG3' was ligated into the SmaI site, thus destroying it and creating a NotI site in the cloning region.

BG341 was then linearized by digestion at its unique NotI site. The linearized plasmid was then alkaline phosphatase treated and purified in an identical manner to that described above for BG368.

Slices of low melt agarose containing either the vector or the insert were diluted with H₂O and melted at 65°C for 2-3 minutes. We ligated aliquots equivalent to 60 ng and 15 ng respectively of 3.8 kb PLA₂ insert and NotI linearized BG341 vector with 400 units of T4 ligase in a total volume of 40 µl of 1X T4 ligase buffer overnight at room temperature. We used 20 µl of this ligation mixture to transform E.coli MC1061 cells. The transformants were grown on LB + ampicillin plates at 37°C overnight. We performed plasmid miniprep analysis using restriction enzymes AatII and NotI to determine orientation. We obtained one transformant in the + orientation, named PLA₂ 3.8 BG341(+). We performed a large scale plasmid preparation of this DNA to obtain supercoiled plasmid.

EXAMPLE 10Expression Of Phospholipase A₂ In
COS-7 African Green Monkey Kidney Cells

We performed DNA mediated transfection

5 using the DEAE/dextran method (L. M. Sompayrac and
K. J. Danna, "Efficient Infection of Monkey Kidney
Cells With DNA of Simian Virus 40" Proc. Natl.
Acad. Sci. USA, 78, pp. 7575-78 (1981)). We trans-
fected 100 mm² tissue culture dishes containing

10 approximately 2 x 10⁶ COS-7 cells (ATCC No. CRL 1651)
with 3 ml each of 10 µg/ml of supercoiled plasmid
from the following constructs: PLA₂ 6.2 BG368 3(+),
PLA₂ 6.2 BG368 8(-), PLA₂ 3.8 BG341 (+) and BG341
(as a control). We transfected five plates with

15 each construct and then pooled the media from the
five plates 72 hours after transfection. Cells from
2 plates were harvested by scraping into 150 µl of
0.36 N H₂SO₄. The plates were then rinsed with
2 x 150 µl of 0.18 N H₂SO₄ containing 150 mm NaCl
20 and the washes combined with the cell suspension.
The cell suspensions were centrifuged at 2,000 xg
for 5 minutes at 4°C and the supernatant recovered.

Phospholipase A₂ activity was assayed in
cell extracts (50 µl aliquots) and media (5 µl) using
25 the ³H-oleic acid labelled E.coli assay described in
Example 3. We found that cells transfected with
PLA₂ constructs in the + orientation relative to the
adenovirus late promoter and, in particular, the
media therefrom, contained significant amounts of
30 phospholipase A₂ activity when compared to untrans-
fected cells and cells transfected with vector
alone.

The table below depicts a comparison of
the levels of expression of phospholipase A₂ in
35 COS-7 cells transfected with various PLA₂
constructs of this invention as well as controls.

-42-

TRANSIENT EXPRESSION
OF INFLAMMATORY PLA₂

	<u>Conditioned Media</u>	<u>PLA₂ (ng/ml)</u>
5	COS cells	<0.03
	COS cells transfected with BG 341 (vector control)	<0.02
	COS cells transfected with PLA ₂ 6.2 BG368 3(+)	1
10	COS cells transfected with PLA ₂ 3.8 BG341(+)	3

15 The expression and secretion of phospholipase A₂ in cells transfected with PLA₂ 3.8 BG341(+) indicated that in the presence of an exogenous promoter, such as the adenovirus major late promoter, the amino acids encoded by nucleotides 2453-2492 can serve as a functional signal sequence.

EXAMPLE 11

20 Construction Of Cell Lines That
Stably Express Phospholipase A₂

25 Because the transfected COS-7 cells described in Example 10 expressed phospholipase A₂ only transiently, we next constructed cell lines that stably expressed the polypeptide. We employed the commercially available expression host CHO DHFR⁻, the dihydrofolate reductase deletion mutant of the Chinese Hamster Ovary cell line.

30 We transfected these cells with a mixture of PLA₂ 3.8 BG341(+) which had been linearized by digestion with restriction enzyme XmnI and pAdD26 (R. J. Kaufman and P. A. Sharp, "Amplification and Expression of Sequences Cotransfected With a Modular Dihydrofolate Reductase Complementary DNA Gene", J. Mol. Biol., 159, pp. 661-21 (1982)) that had been linearized with restriction enzyme StuI in a 9:1 molar ratio to provide the maximum number of PLA₂

gene copies per transfected. pAdD26 is a plasmid which contains a functional DHFR gene. Transfection was achieved by CaPO_4 precipitation. Alternatively transfection may be effected by electroporation or 5 spheroplast fusion.

Following transfection, we incubated the cells for 2 days in nonselective medium α^+ MEM. We then split the cells by diluting 1:10 into selective medium α^- MEM + 10% dialyzed fetal calf serum. Clones 10 were visible after 9 days. After 11 days, cloning rings were inserted on the plates and each clone was trypsinized and transferred to one well of a 48 well microtiter plate. When clones became nearly confluent, we removed the media and assayed it for phospholipase 15 A_2 activity. The cells were expanded in 6 well microtiter plates. We assayed 66 clones and froze in liquid nitrogen the twenty that expressed phospholipase A_2 at the highest level. The five highest expressors were subcloned for amplification in 30 nM 20 methotrexate. These clones or others may be further amplified by growth in higher concentrations of methotrexate.

We also created a second construct, PLA_2 3.8 JODS (Figure 15B), for the expression of phospholipase 25 A_2 in animal cells. In this vector, the phospholipase A_2 coding sequences and the DHFR coding sequences are on the same plasmid. Parent plasmid pJODS (Figure 15A) was digested with AatII and NotI. PLA_2 3.8 BG341(+) was also digested with the same 30 enzymes. Both digests were purified on a low melt agarose gel and the appropriate band excised from the gel. The AatII-NotI fragment from PLA_2 3.8 BG341(+) (containing the promoter elements from BG341 as well as the 3.8 kb PLA_2 insert) was ligated 35 to the AatII-NotI fragment of pJODS (containing the DHFR sequence). We then used the ligated DNA to transform E.coli MC1061 cells. Following mini prep

-44-

analysis to determine the presence of inserts, we performed large scale plasmid preparation from one clone containing the appropriate plasmid. We named the plasmid PLA₂ 3.8 JODS. We then used this plasmid 5 to transform E.coli JA221 cells and prepared CsCl-banded plasmid DNA for use in animal cell transfection. Prior to transfection, PLA₂ 3.8 JODS was linearized with AatII. We routinely used 20 μ g of linearized plasmid to transfect CHO DHFR⁻ cells. 10 Transfections, growth of cells and phospholipase A₂ assays were performed as described above.

EXAMPLE 12

Transfection Of Animal Cells
With PLA₂ Genomic Clones

15 The bacteriophage PLA₂ 8.5 EMBL3 contained approximately 16 kb of human DNA, including the PLA₂ insert which encodes mature inflammatory PLA₂. This phage is modified by standard techniques so that it contains a selectable marker for animal cell 20 expression, such as herpes simplex virus thymidine kinase. Phage particle transfection of mouse L tk⁻ cells is accomplished by the methods of M. Ishiura et al., "Phage Particle-Mediated Gene Transfer to Cultured Mammalian Cells", Mol. Cell Biol., 2, 25 pp. 607-16 (1982).

Specifically, 24 hours prior to transfection, L tk⁻ cells are plated in 100 mm² tissue culture dishes at a density of 5×10^5 - 1×10^6 cells per plate. For each plate, 1 ml of 3×10^7 30 pfu/ml phage particles are coprecipitated with calcium phosphate, pH 6.85, at 25°C for 10 minutes. The precipitate is then absorbed on the L cells for 24 hours at 37°C in 5% CO₂ in air. The cells are then washed with Hepes buffered saline, re-fed with 35 α -MEM and grown for 40 hours. The media is then replaced with HAT media and the cells maintained

until clones appear (about 10 days). Tk^+ clones are assayed for phospholipase A_2 activity.

Alternatively, DNA mediated transfection can be carried out. DNA is prepared from either 5 PLA_2 8.5 EMBL3 or PSQ 130. In the latter case, the 6.2 kb PLA_2 insert is isolated and purified by digestion with HindIII and preparative gel electrophoresis. In the former case, PLA_2 8.5 EMBL3 DNA is linearized by digestion with SalI. Each DNA preparation may 10 then used together with an appropriate selectable marker (e.g., DHFR for CHO DHFR $^-$ cells, thymidine kinase for L tk $^-$ cells) to cotransfect animal cells. DNA-mediated transfection is accomplished by calcium phosphate precipitation or electroporation (G. Chu 15 et al., "Electroporation for the Efficient Transfection of Mammalian Cells With DNA", Nucl. Acids Res., 15, pp. 1311-25 (1987)). Clones positive for the selectable marker are assayed for PLA_2 activity.

EXAMPLE 13

20 Size Determination Of Phospholipase A_2 mRNA From Transformed COS-7 Cells

Cells from three tissue culture dishes (100 mm 2) of COS-7 cells transfected with 10 μ g/ml of supercoiled plasmid from either PLA_2 6.2 BG368 25 3(+), PLA_2 6.2 BG368 8(-), PLA_2 3.8 BG341 (+) or BG341 (as a control) were used to prepare total RNA. The transfected cells were lysed in guanidinium isothiocyanate buffer and total RNA prepared by the method of J. M. Chirgwin et al., "Isolation of Bio- 30 logically Active Ribonucleic Acid from Sources Enriched in Ribonuclease", Biochemistry, 18, pp. 5294-99 (1979). We analyzed 1 μ g and 10 μ g aliquots of total RNA from each transfected cell pool by the Northern blotting technique (H. Lehrach et al., 35 Biochemistry, 10, pp. 4743-51 (1977)) using GeneScreen filters (New England Nuclear, MA) and following the

-46-

manufacturer's instructions. The transferred RNA was hybridized to a ³²P-labelled 1.4 kb OxanI fragment of PSQ 130, which had been labelled by the random priming technique (A. P. Feinberg and

5 B. Vogelstein, "A Technique for Radiolabeling DNA Restriction Endonuclease Fragments", Anal. Biochem., 132, pp. 6-13 (1983); Ibid, "Addendum", Anal. Biochem., 137, pp. 266-67 (1984)). OxanI is the equivalent isoschizomer of commercially available restriction

10 enzymes MstII and Bsu36. All of these enzymes recognize the nucleotide sequence: CCTNAGG. These enzymes cut PSQ 130 at nucleotides 2054-2060 and 3413-3419.

15 The probe hybridized to an 1100 nucleotide mRNA in cells transfected with PLA₂ 3.8 BG341(+). This size is consistent with expected transcription from the adenovirus late promoter and predicted splicing and polyadenylation.

EXAMPLE 14

20 Synthesis Of Human Inflammatory Phospholipase A₂ cDNA

25 Total RNA isolated from cell lines transfected with PLA₂ 3.8 BG341(+) or any other PLA₂ genomic clone which expresses human inflammatory phospholipase A₂ by the above method may be used to obtain a PLA₂ cDNA. Poly A⁺ RNA is isolated from total RNA using oligo dT cellulose chromatography. The poly A⁺ RNA (5-10 µg) is resuspended in H₂O at a concentration of 5 µg/µl and treated with 2.5 mM

30 CH₃HgOH at room temperature for 10 minutes. β-mercaptoethanol is then added to a final concentration of 0.035 M. Synthesis of cDNA is carried out using a cDNA synthesis kit (BRL; catalogue #8267SA) according to manufacturer's directions. The

35 double stranded cDNA is then ligated to linker 35-36:

-47-

5 'AATTCGAGCTCGAGCGCGGCCG3'

3' GCTCGAGCACGCCGGCG5'

using standard procedures. The cDNA is then size selected using Select 4L, 5L, or 6L columns (5 Prime-3 5 Prime Inc., West Chester, PA) according to manufacturer's directions. The size-selected cDNA is then ligated to EcoRI digested λgt10. Aliquots of the ligation reaction are packaged in Gigapack (Stratagene, San Diego, CA) according to the manufacturer's protocol. The packaged phage are then 10 used to infect E.coli BNN102 cells and plated for amplification. PLA₂ cDNA clones are then obtained by screening the resultant library with antisense oligonucleotides from the PLA₂ coding region of 15 PLA₂ 3.8 BG341(+).

Alternatively, PLA₂-specific mRNA is enriched initially by hybrid selection with PLA₂ genomic DNA using any one of the methods described in R. Jagus, "Hybrid Selection of mRNA and Hybrid 20 Arrest of Translation" Meth. Enzymol., 152, pp. 567-72 (1987).

The predicted nucleotide sequence of the PLA₂ coding region of the above-described cDNA is:
AAT TTG GTG AAT TTC CAC AGA ATG ATC AAG TTG ACG ACA
25 GGA AAG GAA GCC GCA CTC AGT TAT GGC TTC TAC GGC TGC
CAC TGT GGC GTG GGT GGC AGA GGA TCC CCC AAG GAT GCA
ACG GAT CGC TGC TGT GTC ACT CAT GAC TGT TGC TAC AAA
CGT CTG GAG AAA CGT GGA TGT GGC ACC AAA TTT CTG AGC
TAC AAG TTT AGC AAC TCG GGG AGC AGA ATC ACC TGT GCA
30 AAA CAG GAC TCC TGC AGA AGT CAA CTG TGT GAG TGT GAT
AAG GCT GCT GCC ACC TGT TTT GCT AGA AAC AAG ACG ACC
TAC AAT AAA AAG TAC CAG TAC TAT TCC AAT AAA CAC TGC
AGA GGG AGC ACC CCT CGT TGC.

Microorganisms and recombinant DNA molecules 35 prepared by the processes of this invention are exemplified by cultures deposited in the In Vitro International, Inc. culture collection, in

-48-

Linthicum, Maryland. These include cultures deposited on July 11, 1988 and identified as:

5 PSQ130/E.coli MC1061
PLA₂ 6.2 BG368 3(+)/E.coli MC1061
PLA₂ 3.8 BG341(+)/E.coli MC1061
PLA₂ 3.8 JODS/E.coli JA221;

and cultures deposited on July 12, 1988 and identified as:

10 PLA₂ 8.5 EMBL3/E.coli LE392.

These deposits were assigned accession numbers IVI 10174-10178, respectively.

15 While we have hereinbefore described a number of embodiments of this invention, it is apparent that our basic constuctions can be altered to provide other embodiments which utilize the processes, polypeptides and DNA sequences of this invention. Therefore, it will be appreciated that the scope of this invention is to be defined by the 20 claims appended hereto rather than by specific embodiments which have been presented hereinbefore by way of example.

CLAIMS

We Claim:

1. A process for purifying an acid stable phospholipase A₂ from a biological source comprising the steps of:

a) extracting said source with an acid;

b) replacing the acid in the extract produced in step a) with a buffer suitable for cation exchange chromatography;

c) contacting said extract with a cation exchange resin to bind the phospholipase A₂ contained in the extract;

d) eluting said phospholipase A₂ from said resin;

e) concentrating said phospholipase A₂;

f) separating said phospholipase A₂ from contaminants by molecular sizing chromatography; and

g) separating said phospholipase A₂ from contaminants remaining after step f) by reverse phase HPLC.

2. The process according to claim 1, further comprising the steps of electrophoresing said phospholipase A₂ and transferring said electrophoresed phospholipase A₂ to a solid support.

3. The process according to claim 2, wherein said electrophoresing is effected by sodium dodecyl sulfate polyacrylamide gel electrophoresis and said solid support is a polyvinylidene difluoride membrane.

4. The process according to claim 1 or 2, wherein said phospholipase A₂ is located within a cell.

-50-

5. The process according to claim 4, further comprising before step a), the step of releasing said phospholipase A₂ from said cell.

6. The process according to claim 5, wherein said biological source comprises human, non-pancreatic cells.

7. The process according to claim 4, wherein said biological source comprises platelets.

8. The process according to claim 1 or 2, wherein said phospholipase A₂ is located extracellularly.

9. The process according to claim 8, wherein said biological source comprises human extracellular fluid.

10. The process according to claim 9, wherein said biological source comprises rheumatoid synovial fluid.

11. The process according to claim 1 or 2, wherein said acid is sulfuric acid having a pH of about 1.

12. A polypeptide comprising the amino acid sequence:

Asn-Leu-Val-Asn-Phe-His-Arg-Met-Ile-Lys-Leu-Thr-Thr-Gly-Lys-Glu-Ala-Ala-Leu.

13. A polypeptide consisting essentially of at least five consecutive amino acids selected from the amino acid sequence:

Asn-Leu-Val-Asn-Phe-His-Arg-Met-Ile-Lys-Leu-Thr-Thr-Gly-Lys-Glu-Ala-Ala-Leu.

-51-

14. The polypeptide according to claim 13, wherein said polypeptide has the sequence:

H₂N-Leu-Val-Asn-Phe-His-Arg-Met-Ile-Lys-Leu-Thr-Thr-Gly-Lys-Glu-Ala-COOH.

15. An oligonucleotide comprising a nucleotide sequence selected from the group consisting of nucleotide sequences which code for the polypeptide according to claim 12 or 13.

16. The oligonucleotide according to claim 15, selected from the group consisting of PLA₂-06, PLA₂-07, PLA₂-08, PLA₂-09, PLA₂-10, PLA₂-11, PLA₂-12, PLA₂-13, and combinations thereof.

17. Phospholipase A₂ purified by the process according to claim 1 or 2.

18. A pharmaceutical composition for eliciting antibodies to acid-stable inflammatory phospholipase A₂ comprising an immunologically effective amount of an immunogen which comprises a polypeptide according to claim 13 coupled to a carrier.

19. A pharmaceutical composition effective as an anti-inflammatory agent comprising a therapeutically effective amount of an antibody selected from the group consisting of antibodies to acid stable inflammatory phospholipase A₂, antibodies to the polypeptide according to claim 12, and combinations thereof.

20. A composition comprising an amount of an antibody selected from the group consisting of antibodies to acid stable inflammatory phospholipase A₂, antibodies to the polypeptide according to claim 12, and combinations thereof, wherein said

-52-

composition is effective to detect the presence of acid stable inflammatory phospholipase A₂ in a biological sample.

21. A method for treating inflammation comprising the step of treating a patient in a pharmaceutically effective manner with a composition according to claim 19.

22. A method for detecting the presence of acid stable inflammatory phospholipase A₂ in a biological sample comprising the step of contacting said sample with a composition according to claim 20.

23. The use of a pharmaceutically effective amount of an antibody to human inflammatory phospholipase A₂ for the treatment of inflammation in mammals.

24. A recombinant DNA molecule comprising a DNA sequence coding for a human inflammatory phospholipase A₂-like polypeptide, said DNA sequence comprising the sequence:

C CAT GGG AAT TTG GTG AAT TTC CAC AGA ATG
ATC AAG TTG ACG ACA GGA AAG GAA GCC GCA CTC AGT TAT
GGC TTC TAC GGC TGC CAC TGT GGC GTG GGT GGC AGA GGA
TCC.

25. A process for producing a human inflammatory phospholipase A₂-like polypeptide comprising the step of culturing a unicellular host transformed with a recombinant DNA molecule according to claim 24.

26. The recombinant DNA molecule according to claim 24, wherein said DNA molecule is PSQ 130.

27. A DNA sequence coding for a human inflammatory phospholipase A₂-like polypeptide, said DNA sequence being selected from the group consisting of:

- (a) the 6.2 kb PLA₂ inserts of PLA₂ 8.5 EMBL3, PSQ 130, and PLA2 6.2 BG368 3(+),
- (b) the 3.8 kb PLA₂ inserts of PLA₂ 3.8 BG341(+) and PLA₂ 3.8 JODS,
- (c) DNA sequences which hybridize to the foregoing PLA₂ inserts and which code on expression for a human inflammatory phospholipase A₂-like polypeptide, and
- (d) DNA sequences which code on expression for a human inflammatory phospholipase A₂-like polypeptide coded for on expression by any of the foregoing DNA inserts and sequences.

28. The DNA sequence according to claim 27, said DNA sequence being selected from the group consisting of a DNA sequence with the formula:

(a) AAT TTG GTG AAT TTC CAC AGA ATG ATC
AAG TTG ACG ACA GGA AAG GAA GCC GCA CTC AGT TAT GGC
TTC TAC GGC TGC CAC TGT GGC GTG GGT GGC AGA GGA TCC
CCC AAG GAT GCA ACG GAT CGC TGC TGT GTC ACT CAT GAC
TGT TGC TAC AAA CGT CTG GAG AAA CGT GGA TGT GGC ACC
AAA TTT CTG AGC TAC AAG TTT AGC AAC TCG GGG AGC AGA
ATC ACC TGT GCA AAA CAG GAC TCC TGC AGA AGT CAA CTG
TGT GAG TGT GAT AAG GCT GCT GCC ACC TGT TTT GCT AGA
AAC AAG ACG ACC TAC AAA AAG TAC CAG TAC TAT TCC
AAT AAA CAC TGC AGA GGG AGC ACC CCT CGT TGC.

29. A recombinant DNA molecule comprising a DNA sequence coding for a human inflammatory phospholipase A₂-like polypeptide, said DNA sequence being selected from the group consisting of:

- (a) the 6.2 kb PLA₂ insert of PLA₂ 8.5 EMBL3,

-54-

(b) DNA sequences which hybridize to the foregoing DNA insert and which code on expression for a human inflammatory phospholipase A₂-like polypeptide; and

(c) DNA sequences which code on expression for a human inflammatory phospholipase A₂-like polypeptide coded for on expression by any of the foregoing DNA inserts and sequences.

30. The recombinant DNA molecule according to claim 29, wherein said DNA sequence is the 3.8 kb PLA₂ insert of PSQ 130.

31. The recombinant DNA molecule according to claim 29, said molecule being selected from the group consisting of: PLA₂ 8.5 EMBL3, PLA₂ 6.2 BG368 3(+), PLA₂ 3.8 BG341(+) and PLA₂ 3.8 JODS.

32. A host transformed with the recombinant DNA molecule according to claim 29, wherein said host is selected from the group consisting of animal cells, insect cells, plant cells, yeast cells and other fungal cells.

33. The host according to claim 32, selected from the group consisting of COS-7 cells and CHO DHFR⁻ cells.

34. A process for producing a human inflammatory phospholipase A₂-like polypeptide comprising the step of culturing the host according to claim 32.

35. A human inflammatory phospholipase A₂-like polypeptide produced by the process according to claim 34.

36. A recombinant DNA molecule comprising a DNA sequence coding for a human inflammatory phospholipase A₂-like polypeptide, said DNA sequence being selected from the group consisting of:

(a) AAT TTG GTG AAT TTC CAC AGA ATG ATC AAG TTG ACG ACA GGA AAG GAA GCC GCA CTC AGT TAT GGC TTC TAC GCC TGC CAC TGT GGC GTG GGT GGC AGA GGA TCC CCC AAG GAT GCA ACG GAT CGC TGC TGT GTC ACT CAT GAC TGT TGC TAC AAA CGT CTG GAG AAA CGT GGA TGT GGC ACC AAA TTT CTG AGC TAC AAG TTT AGC AAC TCG GGG AGC AGA ATC ACC TGT GCA AAA CAG GAC TCC TGC AGA AGT CAA CTG TGT GAG TGT GAT AAG GCT GCT GCC ACC TGT TTT GCT AGA AAC AAG ACG ACC TAC AAT AAA AAG TAC CAG TAC TAT TCC AAT AAA CAC TGC AGA GGG AGC ACC CCT CGT TGC,

(b) DNA sequences which hybridize to the foregoing DNA sequence and which code on expression for a human inflammatory phospholipase A₂-like polypeptide; and

(c) DNA sequences which code on expression for a human inflammatory phospholipase A₂-like polypeptide coded for on expression by any of the foregoing DNA sequences.

37. A host transformed with the recombinant DNA molecule according to claim 36, said host being selected from the group consisting of animal cells, plant cells, yeast and other fungi, and bacteria.

38. A process for producing a human inflammatory phospholipase A₂-like polypeptide comprising the step of culturing the host according to claim 37.

39. A human inflammatory phospholipase A₂-like polypeptide produced by the process according to claim 38.

-56-

40. The recombinant DNA molecule according to any one of claims 24, 29 or 36, said molecule further comprising an expression control sequence, said expression control sequence being operatively linked to said DNA sequence coding for a human inflammatory phospholipase A₂-like polypeptide.

41. A process for producing a human inflammatory phospholipase A₂-like polypeptide comprising the steps of:

(a) transfecting a eukaryotic host with a DNA sequence comprising the 6.2 kb PLA₂ insert; and

(b) culturing said transfected host.

42. The process according to claim 41, wherein said eukaryotic host is selected from the group consisting of a mouse L-cell and a CHO DHFR cell.

43. A human inflammatory phospholipase A₂-like polypeptide produced by the process according to claim 41.

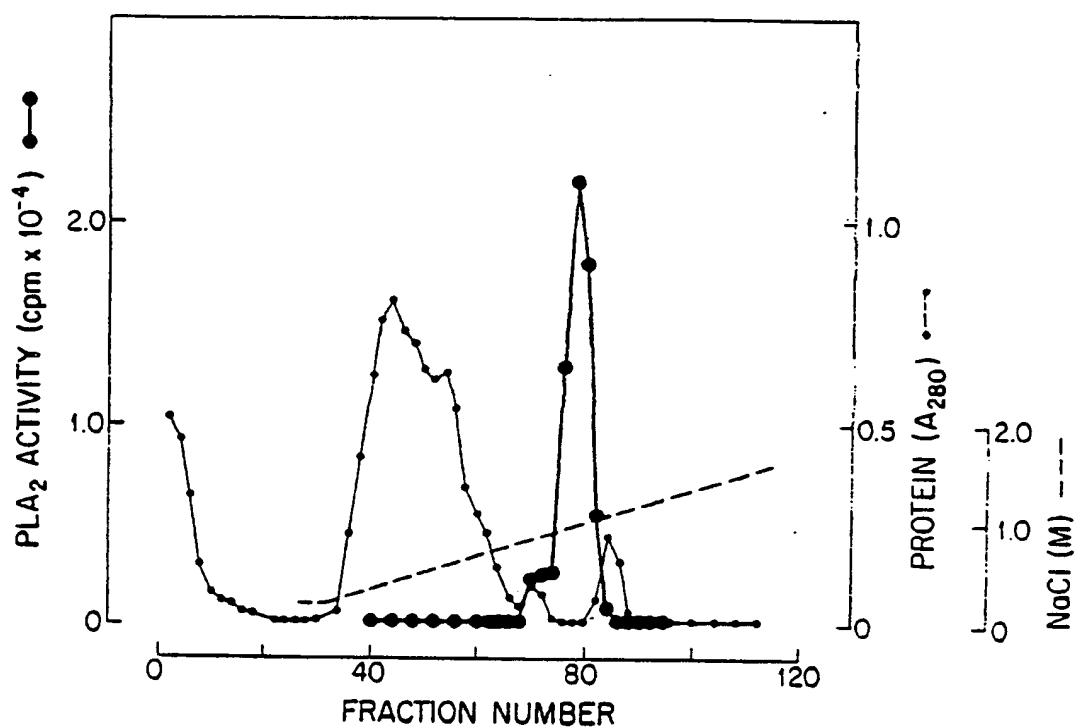
44. The human phospholipase A₂-like polypeptide according to any one of claims 35, 39 or 43, said polypeptide being selected a polypeptide of the formula:

NLVNFHRMIK LTTGKEAALS YGFYGCHCGV
GGRGSPKDAT DRCCVTHDCC YKRLEKRGCG TKFLSYKFSN SGSRITCAKQ
DSCRSQLCEC DKAAATCFAR NKTTYNKKYQ YYSNKHCRGs TPRC.

45. A polypeptide coded for on expression by a DNA sequence selected from the group consisting of the DNA sequences of claim 27 or 28, said polypeptide being essentially free of other proteins of human origin.

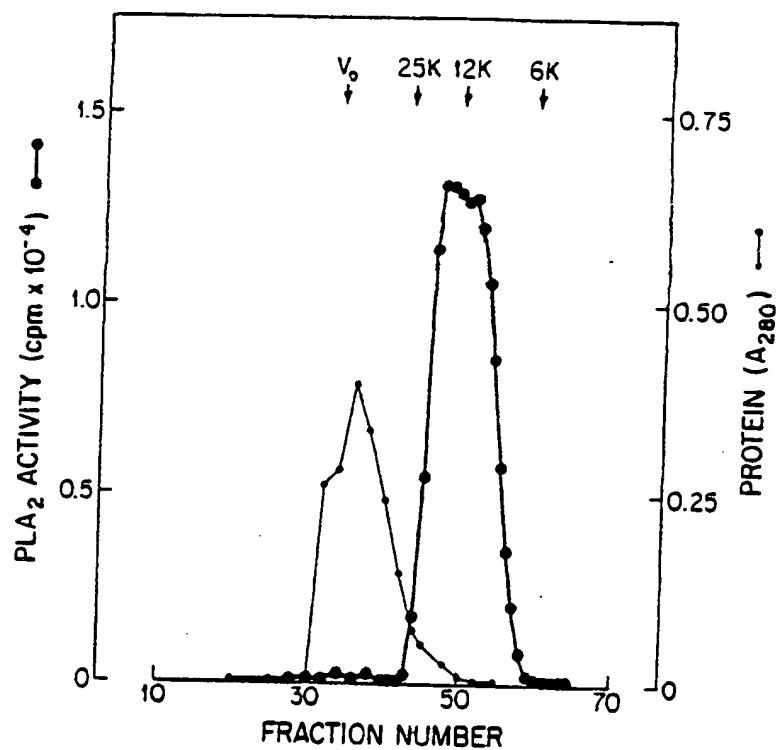
1/23

FIGURE 1



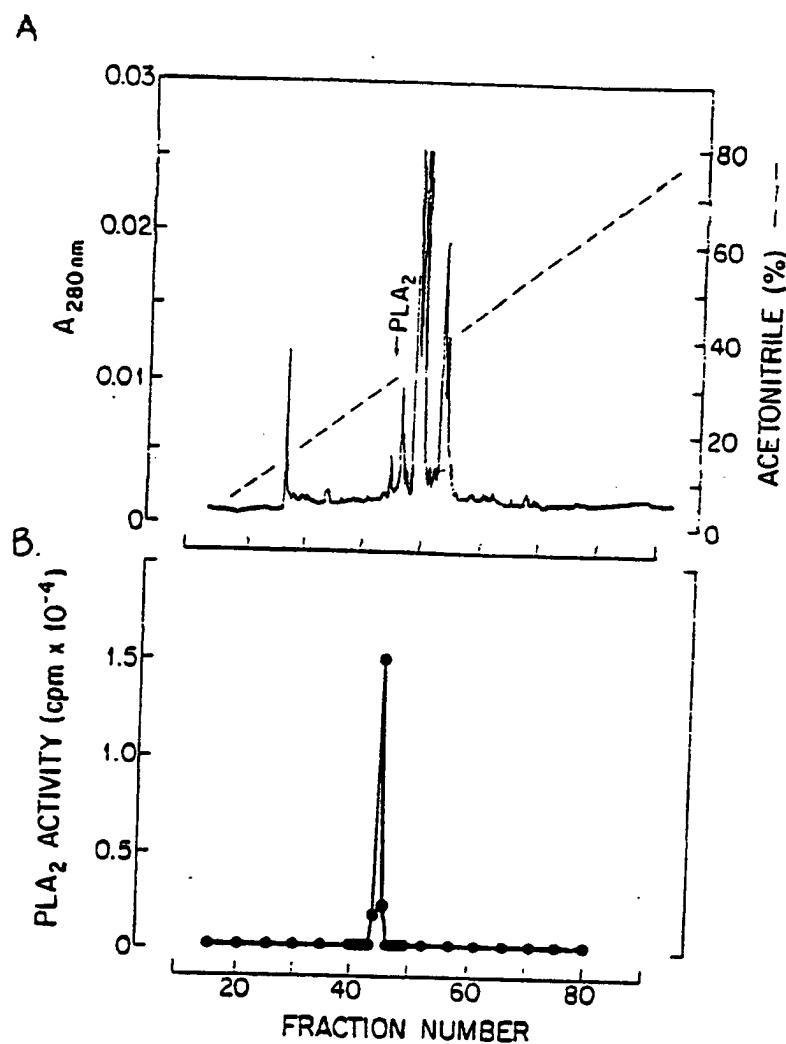
2/23

FIGURE 2



3/23

FIGURE 3



4/23

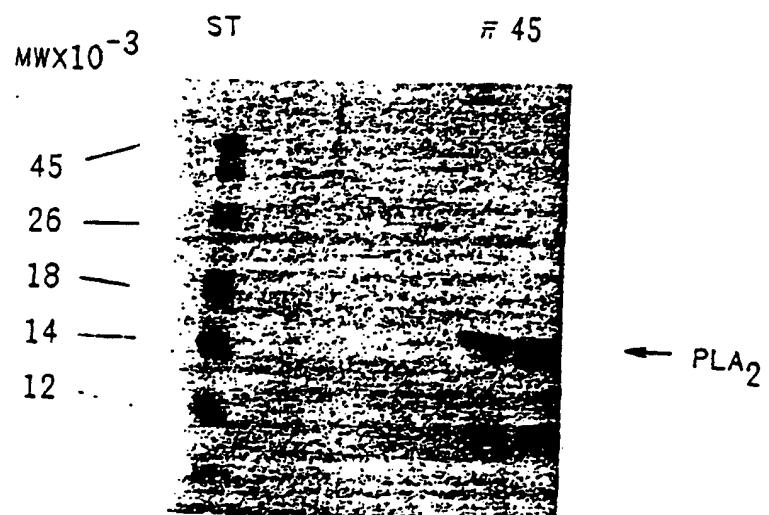
FIGURE 4

STEP	PROTEIN MG	PLA ₂ ACTIVITY		RECOVERY %	PURI- FICATION -FOLD
		TOTAL UNITS	UNITS/ MG		
SONICATION	7510	16	0.0021		
EXTRACTION (PH 1)	6060	26	0.0043		
DIALYSIS (PH 4.5) **	718	1001	1.41	100	11
FAST S CHROMATOGRAPHY	2.5	413	165	41	1300
G-50 GEL FILTRATION	0.1	961	9440	96	>740,000
REVERSE-PHASE HPLC	<0.01	341	146,750	34	>1,100,000

5/23

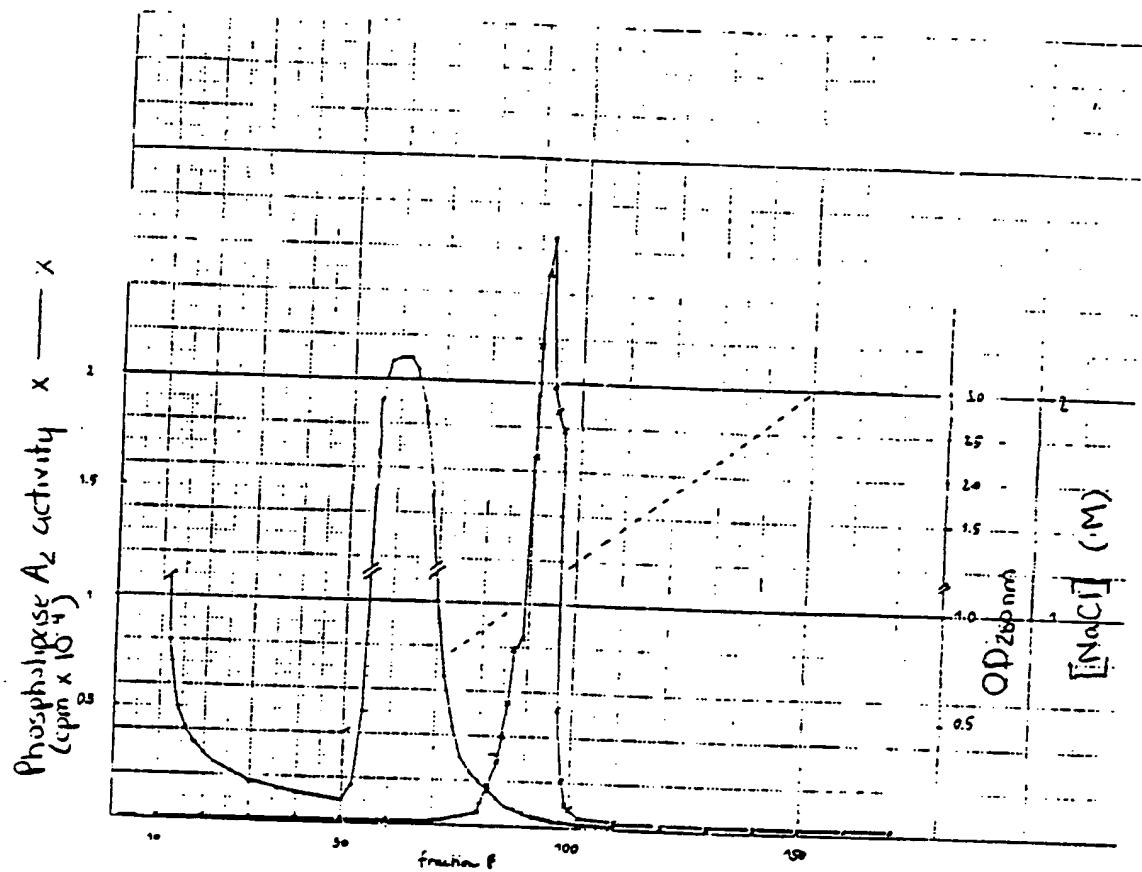
FIGURE 5

COOMASSIE-STAINED PVDF-MEMBRANE



6/23

FIGURE 6



7/23

FIGURE 7

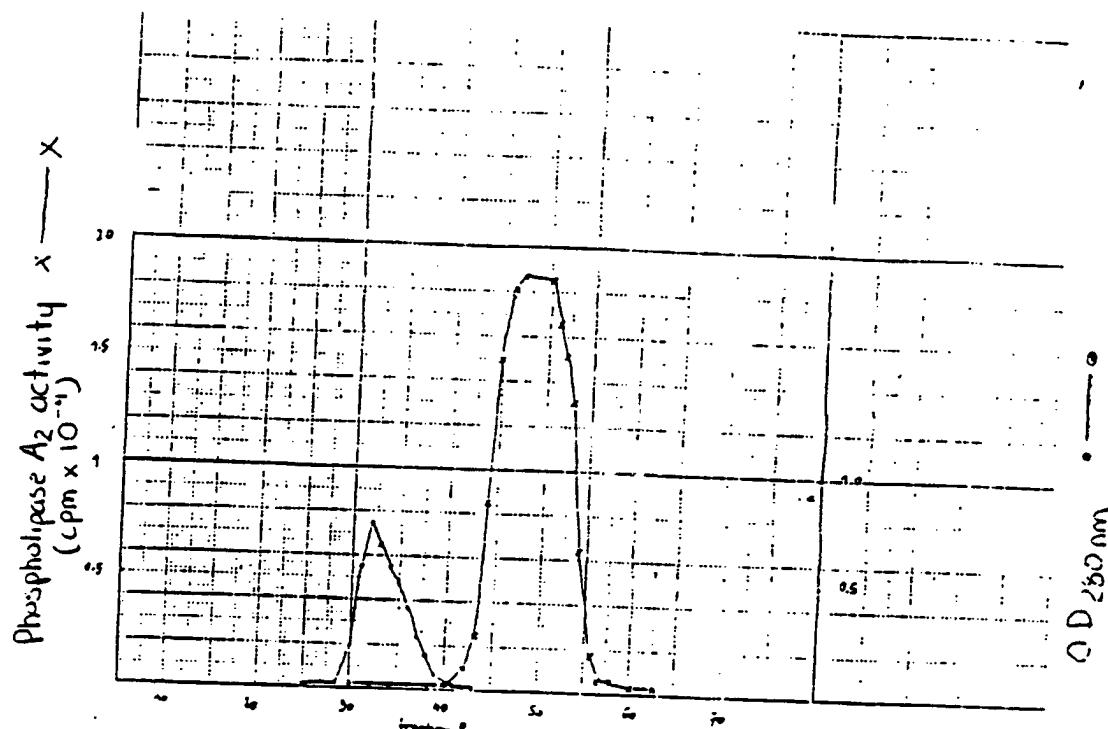
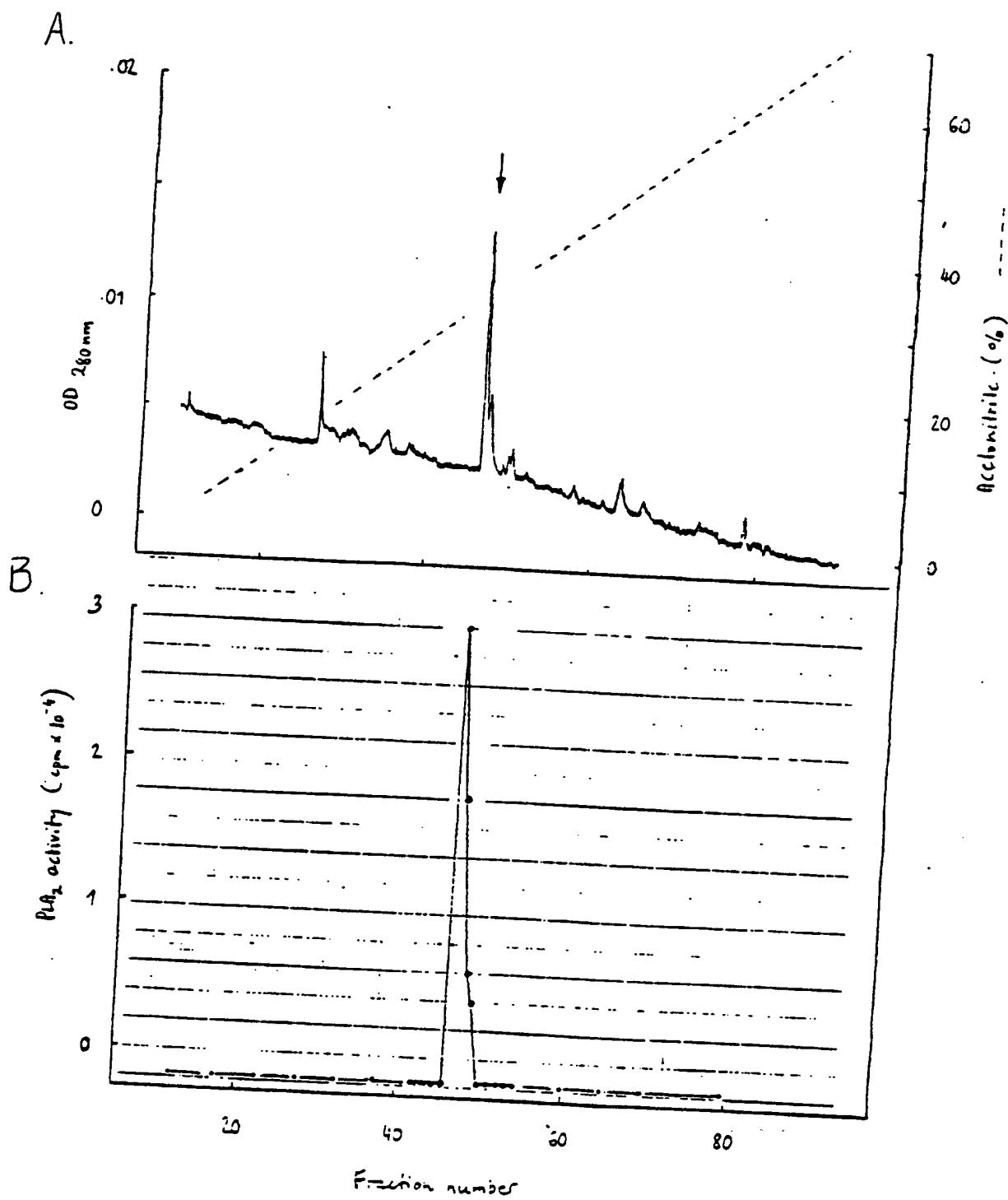


FIGURE 8



9/23

FIGURE 9

PURIFICATION OF HUMAN RHEUMATOID SYNOVIAL FLUID PLA₂

STEP	PROTEIN MG	PLA ₂ ACTIVITY		RECOVERY %	PURI- FICATION -FOLD
		TOTAL UNITS	UNITS/ MG		
H ₂ SO ₄ -EXTRACTION	1932	4.89	0.003	100	
DIALYSIS (pH 4.5)	1582	4.80	0.003	98	
FAST S CHROMATOGRAPHY	4.2	0.36	0.085	7	
G-50 GEL FILTRATION	<0.1	2.03	20.3	42	>8,000
REVERSE-PHASE HPLC	<0.01	2.81	280.7	57	>100,000

10/23

FIGURE 10

COOMASSIE-STAINED PVDF-MEMBRANE

PLA₂ ST MWx10⁻³

- - 45
- - 26
- - 18
- - 14
- - 12

11/23

Figure 11 A

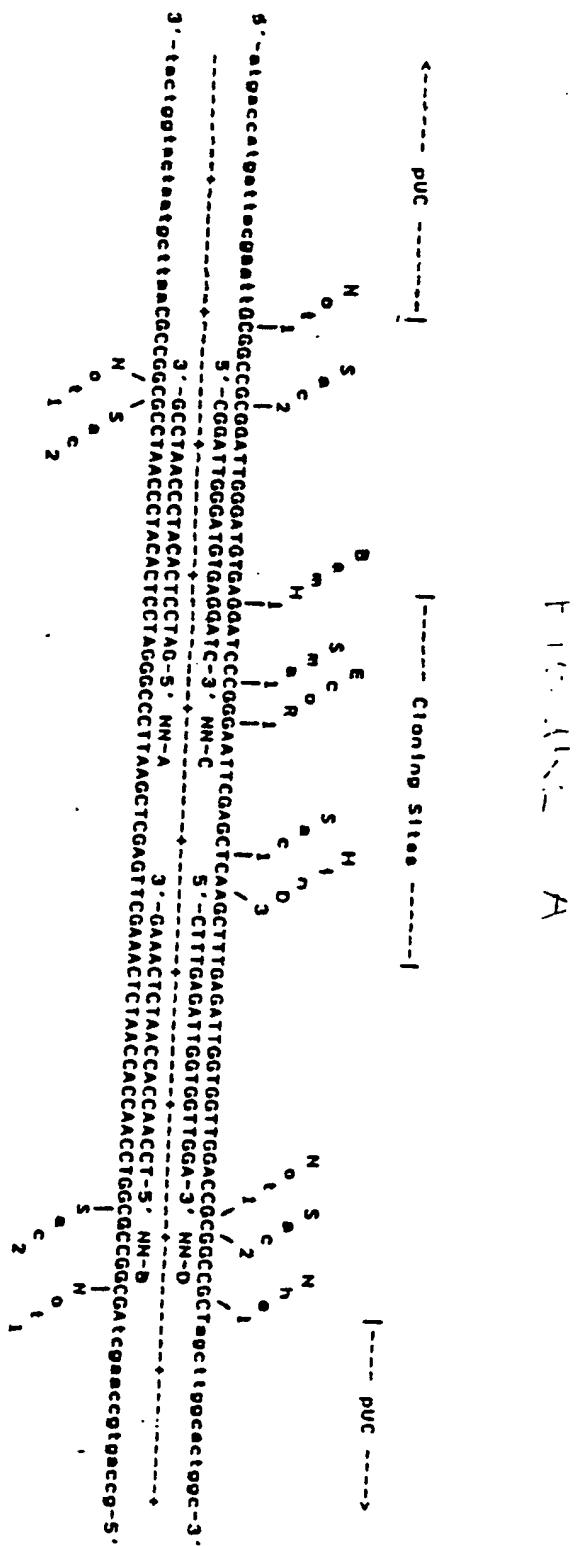
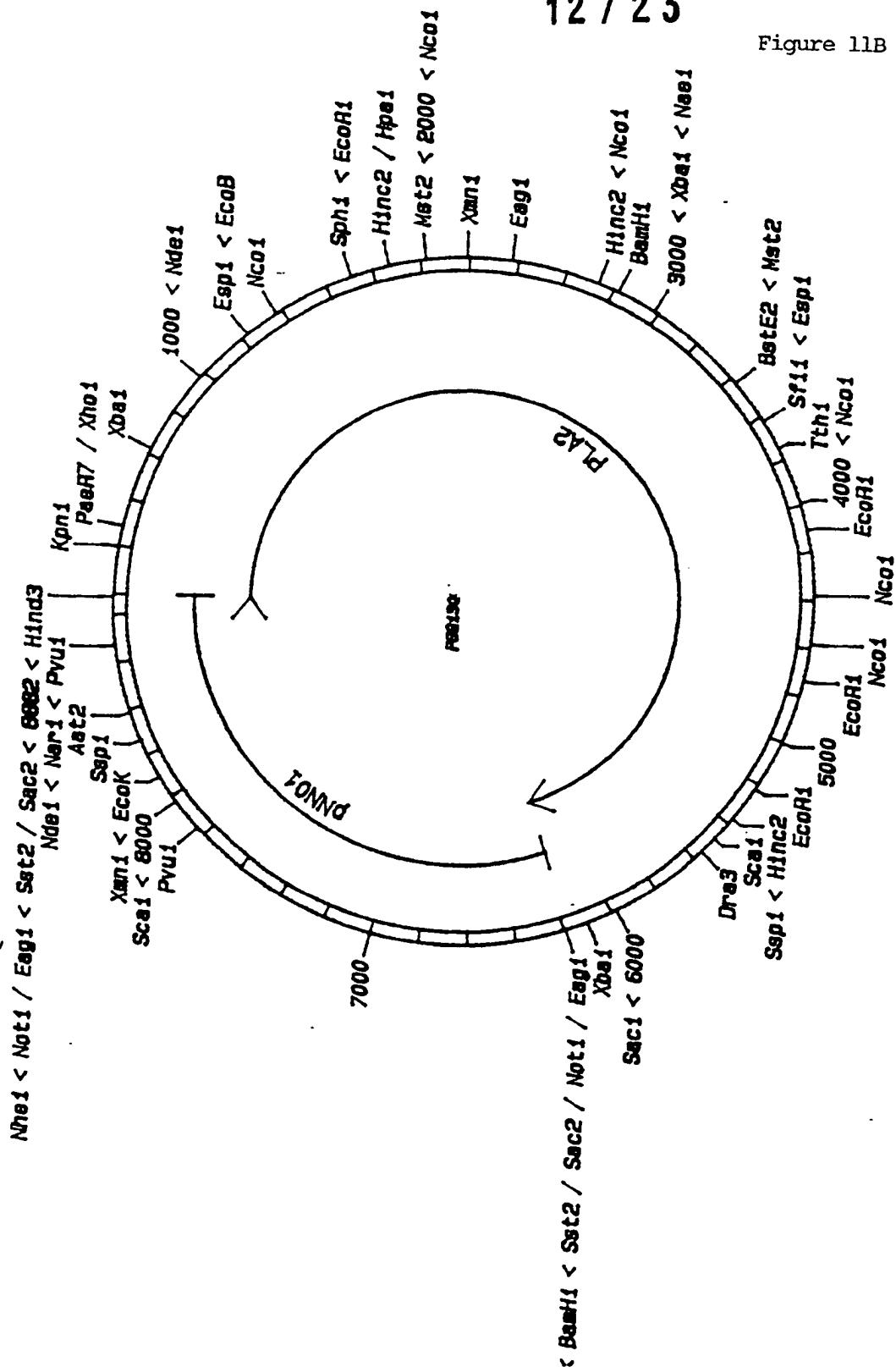


Figure 11B



13 / 23

Figure 12

1 AAGCTTCACCCATCTGCCTCATCTGGCTTGTATGCCCTTGTACTCTGCTGCCCTAGCTGTCATCTGGCTTGT 100
 101 GGATTAGGGCCATCCTAGCTGCCCTAGCTGTCATCTGGCTTGTACTCTGCTGCCCTAGCTGTCATCTGGCTTGT 100
 201 CTCCAAACAGGAACCTGAGGGAGGAGGACACAGTCACCCCTTACACAGTCGAGCCACTGGAGGTAATGGTATTCCTGAGAGGAAACGGGA 200
 301 CATGGCTCTTGGGATAGATTAGGTATGGCTTATGGCTCCAGCAACTGCCATCTCAATTATCATCAGCTTAATACAAACAAAGCTATTTCTCACTCAGCTTAACACTAAAGCTATTTCTCACTCAGCTTAACACTAAAGCT 300
 401 CCCTGTGAGCTGGCAGGACCTCTAGGACAACACTGCTTCCGTGCCATGATTCCTACACCCAGATTGCTTCACTTCTGGTCTACACTAAAGAC 400
 501 GGCTGGGGACTTGGCATCTCATAGTCCCCTTGGGAAACCTGGAAAGTCACCCCTCATACCTAGTGGCTCATGTTTACCACTGAGCTGAGCTGGCTTCACTTCTGGTCTACACTAAAGCT 500
 601 CTCTCCATCCCTGCAAGGGGCTCTAGGACAACAGTCTTCCGTGCCATGAGGTAGGGAAACAGATGGAAATGTTCACAAGGACCAACAGCCACACGGCCCTGCCCACACTGACTTTTACGGACTGGTATCGC 600
 701 AGAGCTTTACTTAAGGGGGTGGAAAGCTAAATTCTTAGCATGTCGGAGGAAATGAAAGATAATTACTTTATGAATTAAAGCTGGACTGGTCA 700
 801 GCCCCGAAGGCTGAAAGGAAAAGGCAACAGATTCACCTGGGAGGATTCAACAGGTGAGGATGGGAATAAAAGTGAAGGGACTGGCTT 900
 901 CGTGGGAACCTCAAGGATAGCCCTCTGGCTTTCAGGTTAGAAGCTCATATGAGACAGGGGGCTGAATCAATGACTTACCTGGGACTGGCTTCAACAGGTGAGGATGGGAATAAGAAGGAAAGCT 900
 1001 TGAGGCCCTGCCCAAGTACTGCGGAAAGGGAAATTCACCCCATTAACCTCTCCCTGGCAATGACTCCACAGCTGAGGCTTGGCAGGGCTG 1000
 1101 GAAACATGGTAGGGCCAGGACATACTTCCCTGTGAATGAATGATTGAGGGCTGAATCAATGACTTACCTAAAGGCCCTTCTTCTATTTCCAAATGCC 1100
 1201 ACATTGACCGAAAGGGACCAATCCTGGCTCAGCAATTGGTAGTCCTGGCAGGTGATGCCAAATGACTCCACAGCTGCAACAGGAGACAGTCTGGCT 1200
 1301 TTAGAGGGATTGGCTGACTGGTGTGATCACACCCAGAGGTGGTTATGGAATTACTCCATGGAAAGACTGCAAAGACTGGCTGAAATGTGT 1300
 1401 TTGGCATCAGCTACTGACACGTAAGCTTCCCAATCCTCAACTCTGTCTGGCAGGTGATGCCAAAGGGATTACCTAGGGTATGGGGACCC 1400
 1501 AATCCCTGAGTCCACCAACTGACCAAGGGCCATCCCTGAGCTGGCTTACCTACCCCAACCTCCAGGGAGGAGCTATTAAGGGGACCCAGGACTCC 1500
 1601 AGAACAAACAGAACGGCTGGGATACAAACTCTGGAGTGGCTCTGAGAGTAAAGGCCAGGAAACTGATGTCTGCAAGGAGCAATTCTGGCTCATT 1600
 1701 CGCTGGCTTTCAGACTCCCTGTTGCTGATGGCATGATTGATCTATGAGGGCTGCTGCTGAGCTGACTGAGTGTGACTGAGTATGTGACTGT 1700
 1801 AGTGTAAAGAGGGATGTTGCCACTATCAGGTAATTACGAGACTGTTGCTGTTAACATGTTATGTTGGCAACTTGTGATGTTGGAGACTCCAAAGCCT 1800
 1901 GAAAGGGGTTAGAAGGGCTAGAACAGAGAGGTTGATGCTTCATTCCTGGAGAAATACTGAGGCCGAGCCCTCCATGGGCTCATGGGAGACTCCAAAGCCT 1900
 2000

14 / 23

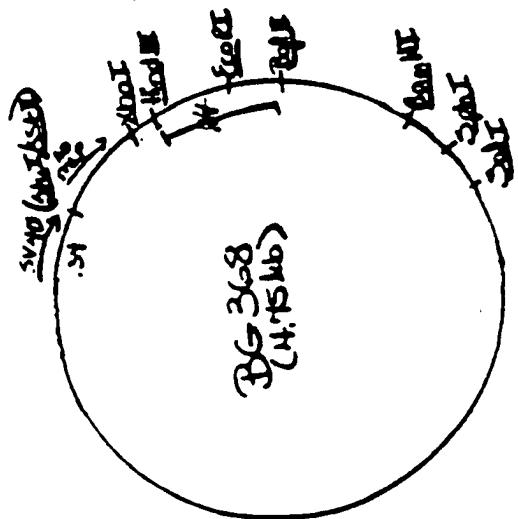
5401	GAAGTCAACTGCTGAGTGTAAAGGCTGACCTGTTGGCTAGAAACAAGACGACCTACAATAMAAAGTACCAACTAACTTCCAATAAACACTG S Q L C E C D K A A T C F A R N K T T Y N K K Y Q Y S N K H C	5500
5501	CAGACGGAGCACCCCTCGTGTAGTCCCTCTTCCGAAACCTTCCACCCAGTGCTGAATTCCCTCTCATACCCCTCCCTACCCCTAACCTAACCA R G S T P R C *	5600
5601	AGTTCCTCTGGCATGGAGAAAGGATCCCTCACCCATCCTAGGGCAGGCCAGGGCCCTTCTATACCCACCCAGAAATGAGACATCAGCAGATTTCCAGC CTTCTACTGCTCTCCACCTCAACTCCGTGCTACTCCGGGCTCTCTCTGAATAAACCAATTAGGAAATCATGTATATGT	5700
5701	GTGTGTGATGGCATGGCTATGCCACTGTTAGCATGACAACAAACAGGGTAGGGTTAAAATACAGGGCTCTGAGTCTGCTCTAGTGTGTCCTGT TGTCCCACCTGGTGGAGGCTGAGGCTGAAGTGTCAAGGAAAGATTGCTTCAGCTCACAGAAAGTTCAGGCAACACAGGGAGACCTTATCTTA	5800
5801	TGTCCCACCTGGTGGAGGCTGAGGCTGAAGTGTCAAGGCAACACAGGGAGACCTTATCTTA AAAAAACAAACAAACAAACGAAAAAAATAGCTTCTGGACTCAGACTGGCTGTCAATCCCGCTCCCCATCTCACTAAGCCCTAGTTCTTCATC 6001	6000
5901	TAGATGGAGATAAACACTGGTGGCTGCATGGCATGTTGACAATTAGAATGACAATGACAATGACAATGACAATGACAATGACAATGACAATGACA 6101	6100
		6172

17 / 23

Figure 13

1 Group I (Bovine) Group II (C. atrox) 6.2 kb PLA ₂ Insert Consensus	50 ALWQFNGMIK CKIPSSEPLL DFNNYGCYCG LGGSGTPVDD LDRCCQTHDN SLVQFETLIM KIAGRSGLLW .YSAYGCYCG WGGHGLPQDA TDRCCFVHDC NLVNFRHMIK LTTGKEAALS .YGFYGCCHCG VGGRGSPKDA TDRCCVTHDC -L--F---I--L--YGC-CG -GG-G-P-D- -DRCC--HD-
51 Group I (Bovine) Group II (C. atrox) 6.2 kb PLA ₂ Insert Consensus	100 CYKQAKKLDs CKVLVDNPYT NNYSYSCSNN EITCSSENNA CEAFIGNCDR CYG. .KATD CNPKTV. .S YTY. SEENG E II.C.GGDDP CGTQI CECDK CYKRLEKRG. CGTKFL. . . SYKFNSSGS RITC.AKQDS CRSQLCECDK CY---K--- C---Y--- -Y--- -I-C--- C---C-CD-
101 Group I (Bovine) Group II (C. atrox) 6.2 kb PLA ₂ Insert Consensus	132 NAAICFSKVP YNKEHKNLDK K.N.C. . . . AAAICFRDNI PSYDNKYWLF PPKDCREEPE PC AAATCFARNK TTYNKKYQYY SNKHCRGSTP RC -AA-CP--- -K--- C---C--- -

Figure 14A



→

- (1) EcoPF
- (2) EcoPF
- (3) EcoPF
- (4) EcoPF
- (5) EcoPF

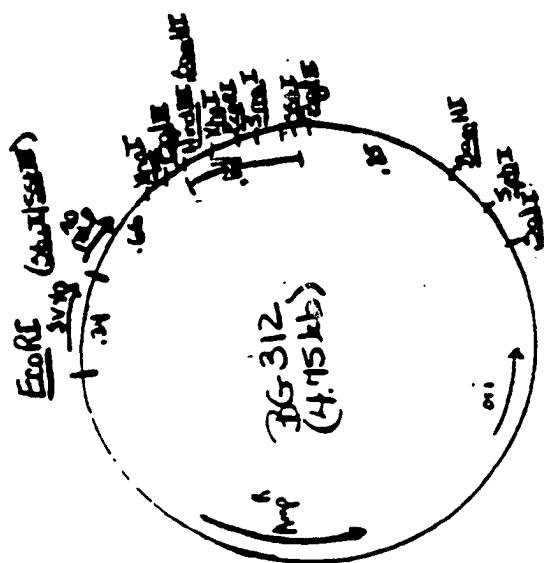
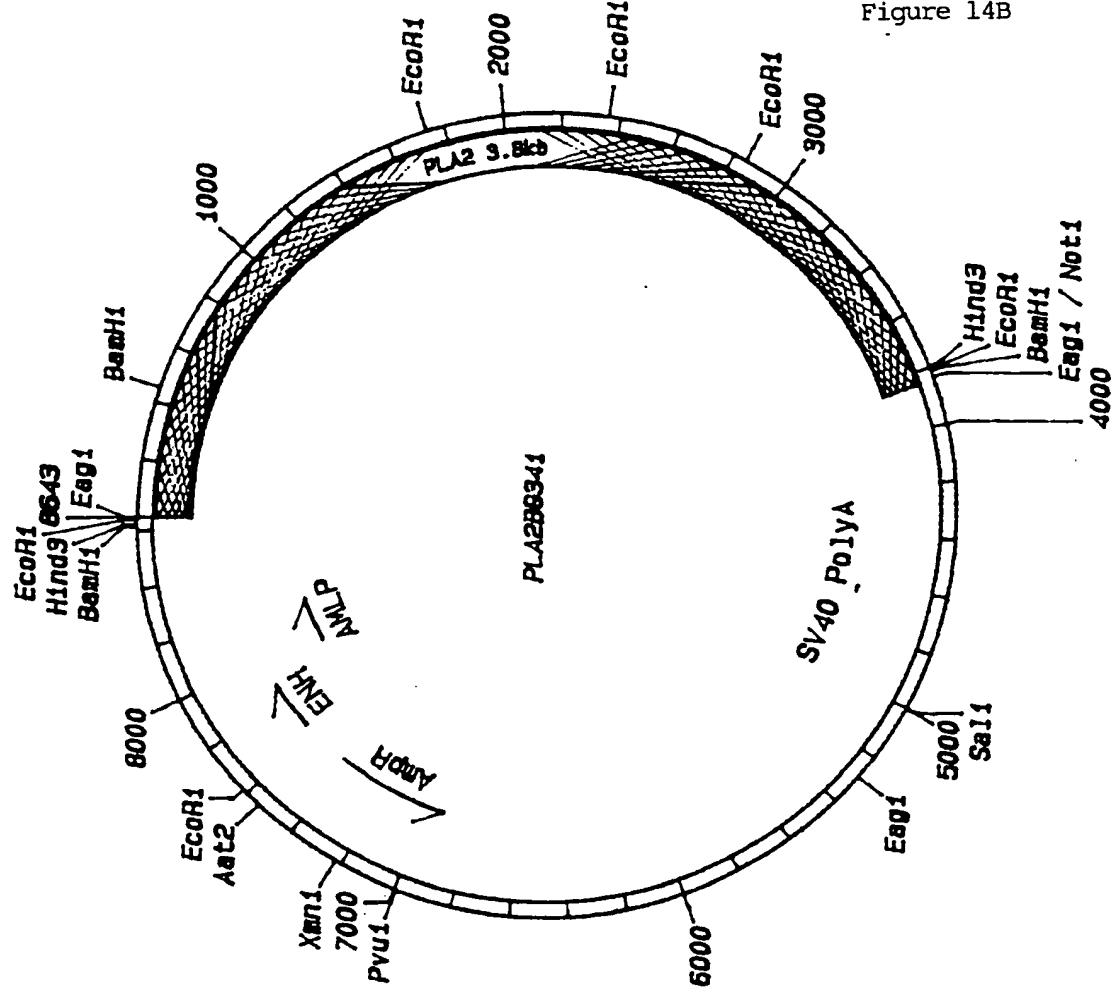
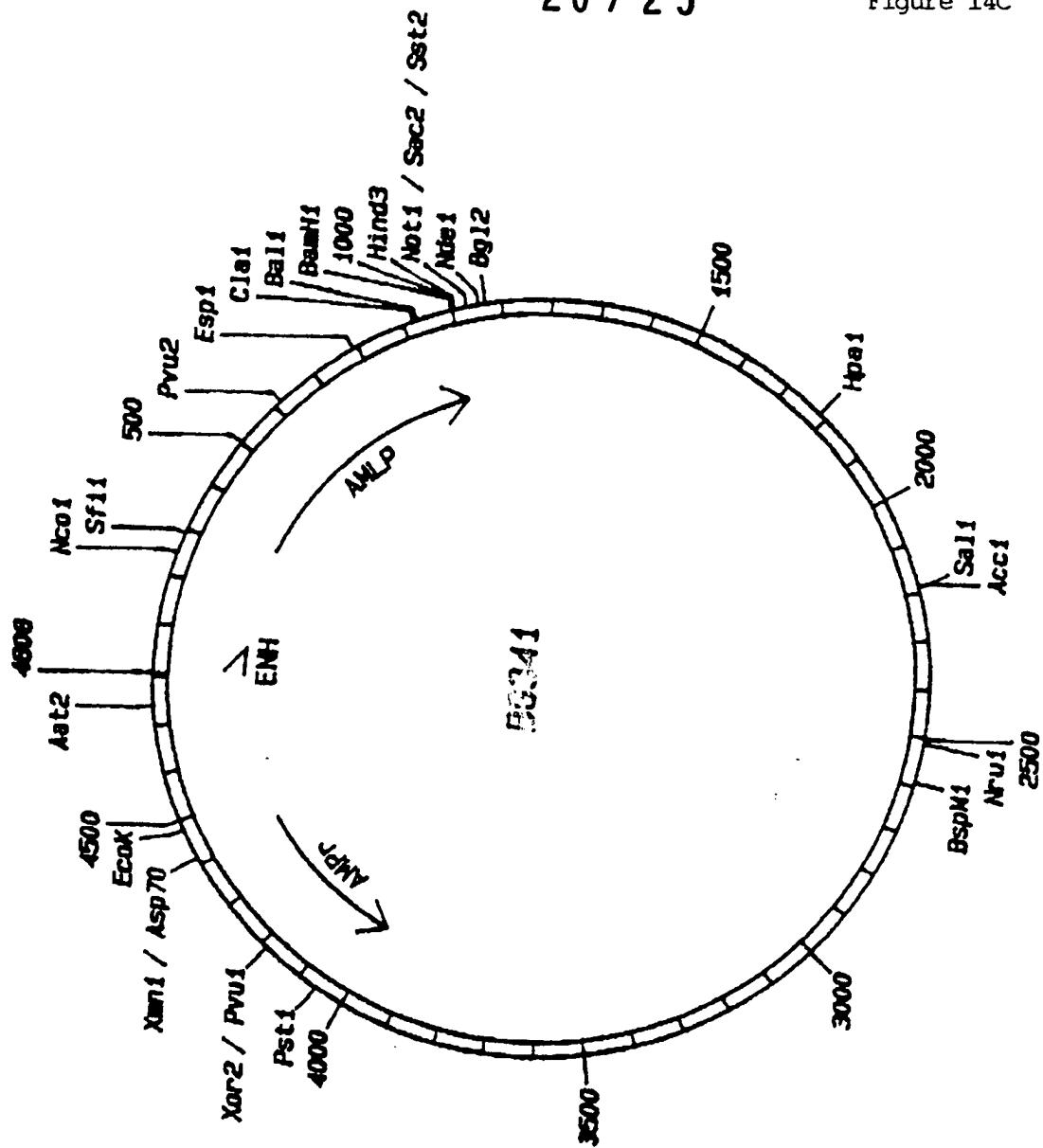


Figure 14B



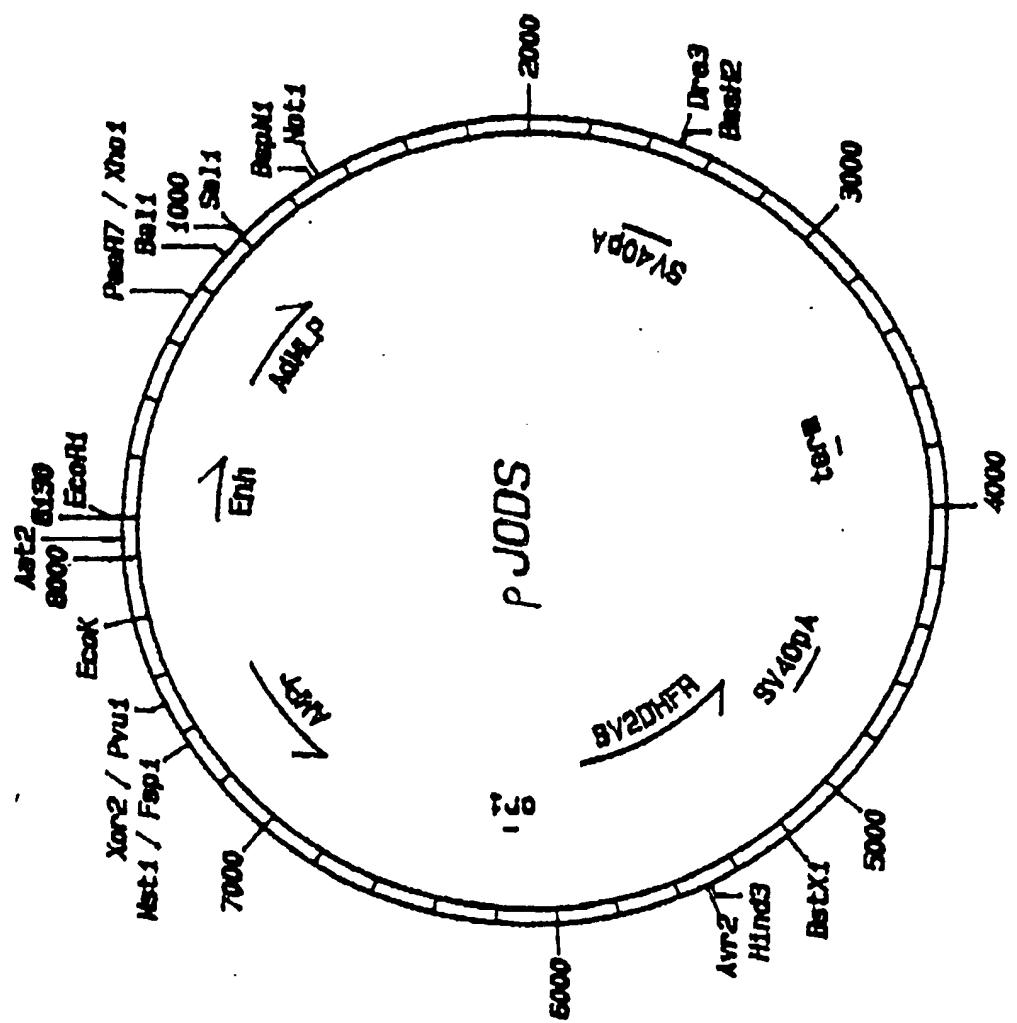
20 / 23

Figure 14C



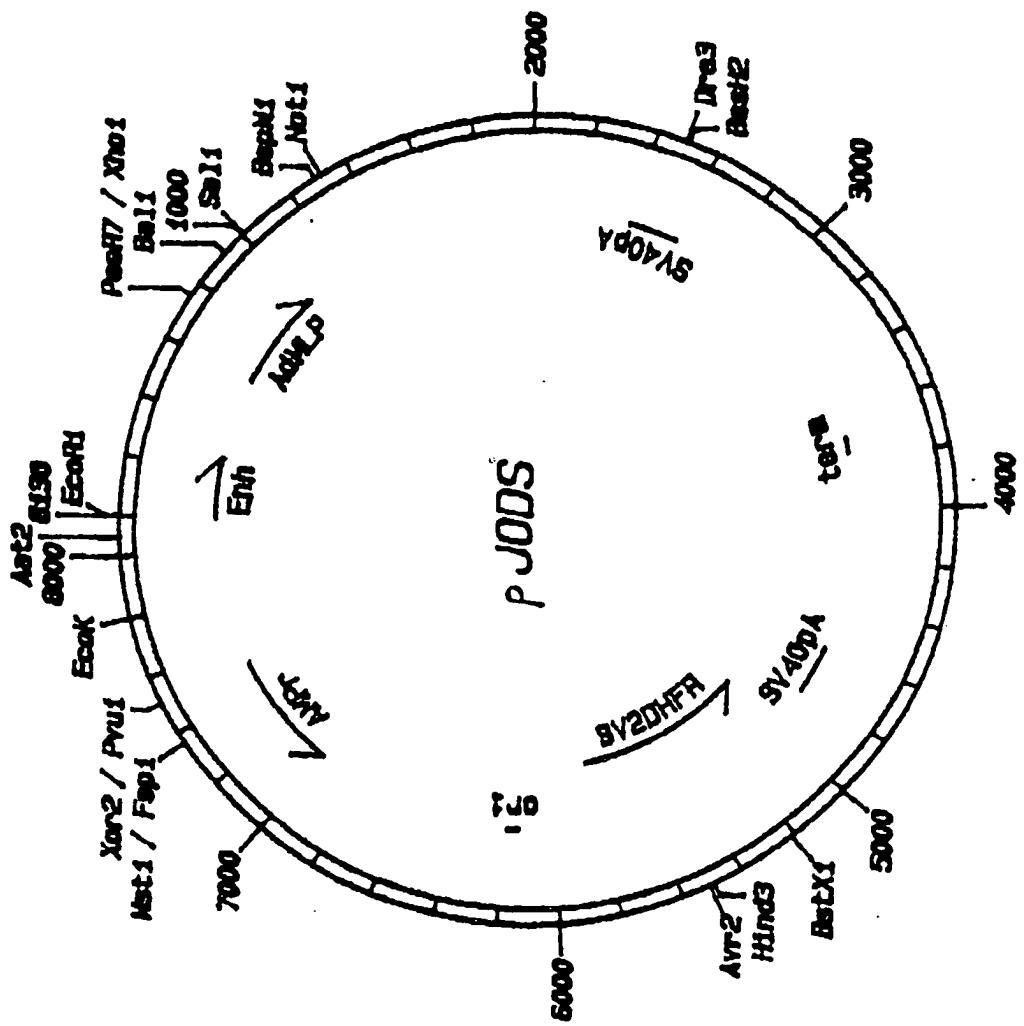
21 / 23

Figure 14 D



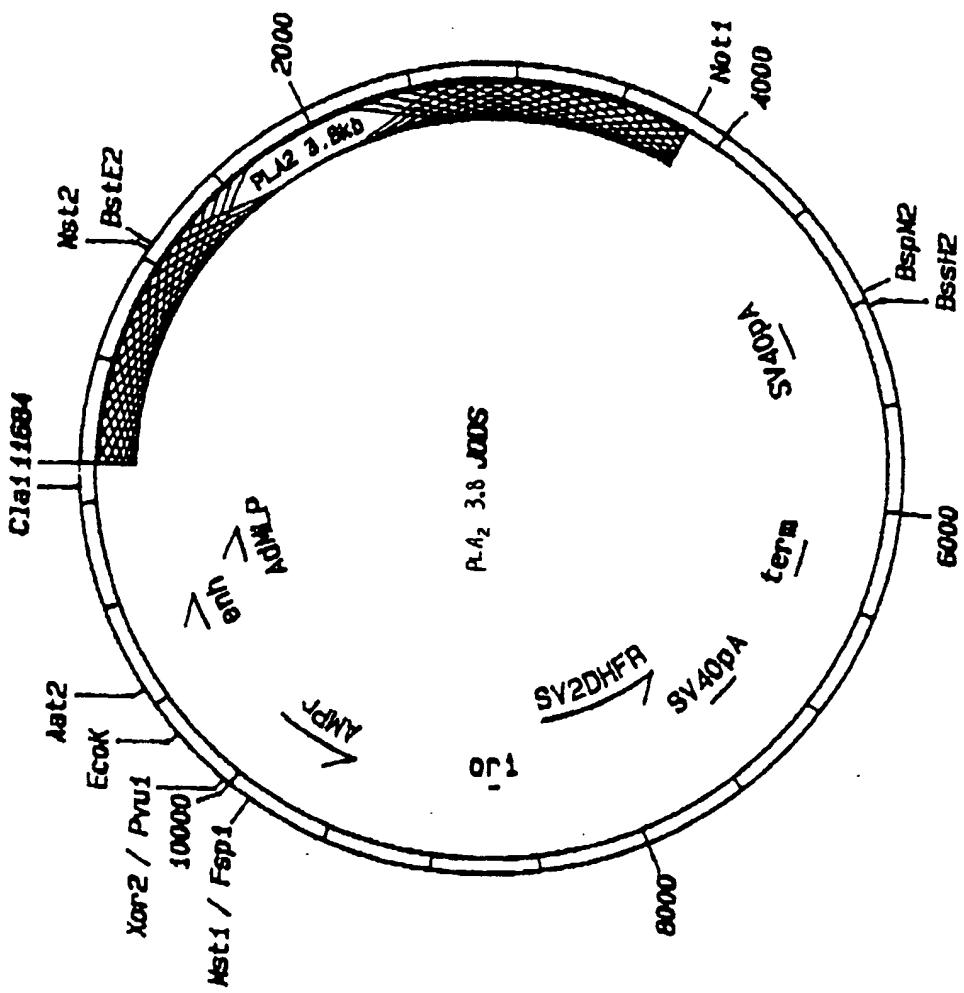
22 / 23

Figure 15 A



23 / 23

Figure 15B



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 89/01418

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC ⁴ C 12 N 9/16, C 07 K 7/08, A 61 K 37/02, A 61 K 39/392,
IPC : C 12 N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
IPC ⁴	C 12 N, C 12 Q, A 61 K, C 07 K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched ⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	Chemical Abstracts, vol. 105, no. 23, 8 December 1986, (Columbus, Ohio, US), R.M. Kramer et al.: "Solubilization and properties of calcium-dependent human platelet phospholipase A2", see page 262, abstract no. 205215g, & Biochim. Biophys. Acta 1986, 878(3), 394-403	17
Y	--	1-11
Y	J. Biochem., vol. 101, no. 5, 1987, M. Hayakawa et al.: "Amino acid composition and NH ₂ -terminal amino acid sequence of rat platelet secretory phospholipase A ₂ 1", pages 1311-1314 see the whole article cited in the application --	1-11
Y	J. Biochem., vol. 102, no. 1, 1987, H.W. Chang et al.: "Purification	1-11
		./.

* Special categories of cited documents: ¹⁰

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"a" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

16th August 1989

Date of Mailing of this International Search Report

13 SEP 1989

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

T.K. WILLIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	and characterization of extra-cellular phospholipase A ₂ from peritoneal cavity of caseinate-treated rat", pages 147-154 . see the whole article cited in the application --	
Y	The Journal of Biological Chemistry, vol. 261, no. 23, 15 August 1986, The American Society of Biological Chemists, Inc., (US), L.A. Loeb et al.: "Identification and purification of sheep platelet phospholipase A ₂ isoforms", pages 10467-10470 see the whole article cited in the application --	1-11
Y	Biochemistry, vol. 25, 1986, American Chemical Society, S. Forst et al.: "Structural and functional properties of a phospholipase A ₂ purified from an inflammatory exudate", pages 8381-8385 see the whole article cited in the application --	1-11
Y	Chemical Abstracts, vol. 92, no. 9, 3 March 1980, (Columbus, Ohio, US), J. Salak et al.: "Isolation of corynebacterial enzymes from cultivation media by gel filtration and ion-exchange chromatography", see page 313, abstract no. 72356t, & Toxicon 1979, 17(6), 655-8 --	1-11
A	Chemical Abstracts, vol. 103, no. 11, 16 September 1985, (Columbus, Ohio, US), T.L. Hazlett et al.: "Affinity chromatography of phospholipase A ₂ from Naja naja naja (Indian cobra) venom", see page 272, abstract no. 83963k, & Toxicon 1985, 23(3), 457-66 --	
A	Chemical Abstracts, vol. 103, no. 1, 8 July 1985, (Columbus, Ohio, US), M.M. Rakhimov et al.: "Biospecific adsorption chromatography of phos- ./.	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

pholipase A₂ from different sources",
see page 245, abstract no. 2623z,
& Prikl. Biokhim. Mikrobiol. 1985,
21(2), 190-8

A J. Biochem., vol. 99, no. 3, 1986,
O. Ohara et al.: "Dog and rat
pancreatic phospholipases A₂:
Complete amino acid sequences
deduced from complementary DNAs",
pages 733-739

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers ... 21 .. because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): Methods for treatment of the human
or animal body by surgery or therapy,
as well as diagnostic methods

2. Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this International application as follows:

1. As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application.

2. As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3. No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

The additional search fees were accompanied by applicant's protest.

No protest accompanied the payment of additional search fees.